

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents
United States Patent and Trademark
Office
Box PCT
Washington, D.C. 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 22 August 2000 (22.08.00)	
International application No. PCT/IL00/00048	Applicant's or agent's file reference 122177.9 LK
International filing date (day/month/year) 25 January 2000 (25.01.00)	Priority date (day/month/year) 25 January 1999 (25.01.99)
Applicant WILLNER, Itamar et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
19 July 2000 (19.07.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).


The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer Claudio Borton Telephone No.: (41-22) 338.83.38
---	--

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 122177.9 LK		FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/IL00/00048	International filing date (day/month/year) 25/01/2000	Priority date (day/month/year) 25/01/1999	
International Patent Classification (IPC) or national classification and IPC G01N33/48			
Applicant YISSUM RESEARCH DEVELOPMENT COMPANY OF THE...et al			
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 13 sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of sheets.</p>			
<p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"> I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input checked="" type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability IV <input checked="" type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input type="checkbox"/> Certain defects in the international application VIII <input checked="" type="checkbox"/> Certain observations on the international application 			
Date of submission of the demand 19/07/2000		Date of completion of this report 26.04.2001	
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465		Authorized officer Thumb, W Telephone No. +49 89 2399 7350	



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL00/00048

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

Description, pages:

1-43 as originally filed

Claims, No.:

1-78 as originally filed

Drawings, sheets:

1/29-29/29 as originally filed

Sequence listing part of the description, pages:

1-4, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL00/00048

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. 1-3, 12, 13, 14, 43-46 (all in part), 21-26, 53-58, 27-34, 59-66 (all entirely).

because:

- ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):
- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☒ no international search report has been established for the said claims Nos. 1-3, 12, 13, 14, 43-46 (all in part), 21-26, 53-58, 27-34, 59-66 (all entirely).

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

- ☐ the written form has not been furnished or does not comply with the standard.
- ☐ the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of invention

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL00/00048

1. In response to the invitation to restrict or pay additional fees the applicant has:

- ☐ restricted the claims.
- ☒ paid additional fees.
- ☐ paid additional fees under protest.
- ☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

- ☐ complied with.
- ☐ not complied with for the following reasons:

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

- ☐ all parts.
- ☒ the parts relating to claims Nos. 1-3, 12, 13, 14, 43-46 (all in part), 4-11, 15-20, 35-42, 47-52, 67-73, 74-78.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	10, 11, 14, 18, 35-42, 50, 67-73, 74-76
	No:	Claims	1-9, 12, 13, 15-17, 19, 20, 43-49, 51, 52, 77, 78
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-20, 35-52, 67-78
Industrial applicability (IA)	Yes:	Claims	1-20, 35-52, 67-78
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

29/ PRTS

09/889936

JCT/Rec'd PCT/PTO 25 JUL 2001

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IL00/00048

Re Item IV

Lack of unity of invention

The application lacks unity within the meaning of Rule 13.1 PCT for the following reasons:

The broadest possible single general inventive concept underlying the present application can be seen in using a specific binding molecule directed against a small molecule to be detected, e.g. trinitrotoluene (TNT) or dinitrotoluene (DNT), in a piezo-electric micro-gravimetric assay. These technical features are included in all claims of the present application.

EP-A-0 453 224 discloses a chemical sensor, which is based on a quartz oscillator, which has a high specificity due to the use of antibodies which are specifically related to a particular chemical, such as TNT or DNT (column 2, lines 20-39). In operation, the crystal oscillator is exposed to water contaminated with the chemical to be detected (column 4, lines 23-25).

WO-A-98/57141 discloses a method to detect an analyte from air, including the steps of extracting the analyte with a solvent and transporting the collected liquid sample to an analytical detection system (page 2, lines 14-31). The analyte is preferably a component of explosives or mines, such as TNT (page 4, lines 23-26). The detection system comprises a flow cell, a quartz crystal, and a surface antibody coating, the antibody specific for and binding to the analyte (page 6, lines 1-7).

Bofors-Schnauzer, "Detection of abandoned land mines", EUREL International conference, 7-9 October 1996, Conference publication No. 431 (page 35, column 2, paragraph 10 - column 2, paragraph 2) also discloses the use of oscillating quartz crystals and antibodies specific for TNT in a biosensor.

Therefore the single general concept underlying the present application is not novel and cannot be considered as being the single general inventive concept within the meaning of Rule 13.1 PCT.

In addition, features relating to the components of the apparatus or systems of the present application (i.e. use of a flow through cell, means to prepare the sample, electronic means to drive the piezoelectric crystal, electronic means to detect changes in the resonant frequency of said crystal and a sensing member bearing a capturing

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IL00/00048

agent on its surface) can also not be considered as being special technical features within the meaning of Rule 13.2 PCT, as they are already disclosed in WO-A-98/57141, page 4, line 24 - page 6, line 21 and page 9, lines 10-31, as well as in Bofors-Schnauzer, "Detection of abandoned land mines", EUREL International conference, 7-9 October 1996, Conference publication No. 431 (page 35, column 2, paragraph 10 - column 2, paragraph 2).

Hence, it is considered that the following separate inventions or groups of inventions are not so linked as to form a single general inventive concept:

1. Claims 1-3, 12, 13, 14, 43-46 (all in part) and 4-11, 15-20, 47-52 (all entirely)

Apparatus and methods for detecting explosives providing :

- as sensing surface carrying moieties or residues of TNT or DNT bound to an antibody.

2. Claims 1-3, 12, 13, 14, 43-46 (all in part) and 21-26, 53-58 (all entirely)

Apparatus and methods for detecting explosives providing :

- as sensing surface carrying moieties or residues of TNT or DNT,
- a reagent for mixing with the sample prior to introduction in the cell comprising an antibody.

3. Claims 1-3, 12, 13, 14, 43-46 (all in part) and 27-34, 59-66 (all entirely)

Apparatus and methods for detecting explosives providing :

- as sensing surface carrying moieties or residues of TNT or DNT,
- a reagent for mixing with the sample prior to introduction in the cell comprising an antibody,
- a filtration system to remove unbound antibodies prior to introduction in the cell.

4. Claims 1-3, 12, 13, 14, 43-46 (all in part) and 35-42, 67-73 (all entirely)

Apparatus and methods for detecting explosives providing :

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IL00/00048

- a reagent for mixing with the sample prior to introduction in the cell comprising an antibody conjugated to an enzyme,
- a sensing surface carrying an antibody capable of binding the enzyme,
- a filtration system to remove unbound antibody-enzyme complexes,
- further reagents reacting with the enzyme to form an insoluble product.

5. Claims 74-78

A protein (antibody) able to bind TNT or DNT described as for use in a piezoelectric micro-gravimetric assay.

Pursuing the provisions of Rule 66.1e) no international preliminary examination is carried out for claims relating to subject-matter for which no international search report was established (i.e. inventions 2 and 3 as defined above).

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The numbering of the individual inventions is according to item IV above.
2. Reference is made to the following documents:

D1: WO 97 04314 A

D2: BRINK S -A: 'Bofors Schnauzer-a biosensor for detection of explosives'
EUREL INTERNATIONAL CONFERENCE. THE DETECTION OF
ABANDONED LAND MINES: A HUMANITARIAN IMPERATIVE SEEKING A
TECHNICAL SOLUTION, 7 October 1996, pages 33-36.

D3: WO 98 57141 A

D4: U. NARANG ET AL.: 'Multianalyte detection using a capillary-based flow
immunosensor.' ANALYTICAL BIOCHEMISTRY, vol. 255, no. 1, 1 January
1998 (1998-01-01), pages 13-19.

D5: D. FETTEROLF ET AL.: 'An enzyme-linked immunosorbent assay (ELISA)

for trinitrotoluene (TNT) residue on hands.' JOURNAL OF FORENSIC SCIENCES, vol. 36, no. 2, (1991), pages 343-349.

D6: PATENT ABSTRACTS OF JAPAN vol. 014, no. 034 (C-679), (1990) & JP 01 269485 A

D7: US-A-4 999 284

3. Invention 1

3.1 Novelty

3.1.1 Document D1 discloses a system for determining binding between two members of a recognition pair, comprising:

- (a) a probe comprising a piezoelectric crystal and one or more plates carried on the surface of said crystal, said plates having immobilized thereon a first member of the recognition pair and the second member bound thereto, whereby dissociation of the two leads to a change of the probes resonance frequency,
- (b) a vessel for holding a liquid, the probe being immersed in the liquid to allow release of the second member, a priori bound to said first member, into said liquid, and
- (c) electric or electronic circuitry for measuring the resonance frequency of said crystal (page 7, line 12 - page 8, line 3; figure 2).

The immobilized member may be an immobilized analyte molecule or a molecule with a similar binding specificity to said second member as said analyte.

Preferable, the immobilized analyte molecule has a lower binding affinity to said second member than the analyte (page 11, line 28 - page 12, line 5).

Said apparatus can be used in a method to detect an analyte in a liquid medium (page 8, line 23 - page 9, line 15).

The sensitivity of the method can be increased by the use of a molecule, moiety or complex, which is complexed or bound to said second member ("amplifier group") (page 17, lines 10-22). Said amplifier group can be an antibody directed against the first antibody (figure 5; page 25, line 26 - page 26, line 12), or avidin conjugated to the antibody via a biotin affinity group (figure 6C).

As an example, a dinitrophenol antigen monolayer electrode with bound DNP-monoclonal antibodies (Ab) for 2,4 dinitrophenol analysis in a sample is

described, where displacement of the antibody, pre-immobilized on the antigen monolayer electrode surface, is used for detection of an antigen in a sample (see point 2.4 on page 32).

Document D1 is therefore prejudicial to the novelty of claims 1-9, 12, 13, 15-17, 19, 20, 43-49, 51, and 52 in the sense of Article 33(2) PCT.

3.1.2 Document D2 discloses a biosensor, comprising a quartz oscillator having antibodies, which are sensitive to TNT, attached to the surface. If the solvent of the sample contains TNT, some of the antibodies release from the surface, which results in a change of the own-frequency of the oscillator due to the change in mass (page 35, column 1, line 43 - column 2, line 4).

Hence, document D2 is novelty-destroying for the subject-matter of claims 1-3, 12, and 43-46 within the meaning of Article 33(2) PCT.

3.2 Inventive step

3.2.1 Claim 14 differs from the teaching of D1 in that a flow system for propelling a medium into a testing cell is included in the apparatus.

The underlying objective problem may therefore be seen in providing a convenient means to transfer a sample to a testing cell.

Although document D1 does not explicitly disclose such a flow system, it is noted that piezoelectric immunoassaying allows stationary and flow analysis of a sample (page 4, lines 15-17). An example is given in document D3, wherein a biosensor system for the detection of explosives, e.g. TNT, is disclosed, using piezoelectric determination of the analyte, and comprising a flow cell, in which the sample solution passes over the surface of an oscillating quartz crystal (page 9, lines 10-14). It would therefore be obvious for the skilled person to include a flow system into the apparatus of document D1 (see item 3.1.1 above) as a normal design feature well known in the art.

Claim 14 thus does not meet the requirements of Article 33(3) PCT.

3.2.2 Claims 10, 11, 18, and 50 differ from the teaching of D1 in that monoclonal antibody 5B3 is used in a piezoelectric immunoassay. According to the description of the present application, said antibody has a high specificity for TNT

(page 22, point 1.6).

The underlying problem may therefore be seen in providing an alternative antibody to be used on an apparatus or in a method for determination of small molecular analytes, especially explosives.

Antibodies selectively binding to TNT are known in the art (see for example document D4, page 14, column 2, line 18; document D5, page 344, lines 44-45; document D6, last paragraph). Since the use of the antibody of the present application does not seem to be associated with a surprising technical effect it is considered as being an mere selection among a number of known possibilities not involving an inventive activity (see also PCT Guidelines, IV-8.8(C1)(i)). Claims 10, 11, 18 and 50 therefore do not meet the requirements of Article 33(3) PCT.

4. Invention 4

4.1 Claims 1-3, 12, 13, and 43-46 do not meet the requirements of Article 33(2) PCT (see item 3.1.1 and 3.1.2 above).

Claim 14 is not considered to be inventive in the sense of Article 33(3) PCT (see item 3.2.1 above).

4.2 Inventive step

4.2.1 Document D7 discloses a quartz crystal-based biosensor, which is coated with an anti-analyte antibody. The sensor is contacted with the sample solution and, after binding of the analyte, it is contacted with a conjugate comprising an anti-analyte antibody and an enzyme. A substrate for the enzyme is added and an insoluble reaction product accumulates on the surface of the sensor, thereby leading to a change of mass and hence a change in resonant frequency, as measured by an external circuit (column 3, line 50 - column 4, line 11).

The subject-matter of claim 35 differs from the teaching of D7 in that the sensor is coated with capturing agents specific for the analyte-binding, neutralising agent, and in that a filter for removing unbound reagents is included in the system.

The underlying objective problem to be solved may therefore be seen in providing a quartz oscillator-based immunoassay having an alternative set-up of components than the one disclosed in D7.

However, changing the order of capturing agents and binding moieties when

coating a solid support is a standard procedure in the art and well known to the person skilled in the art working in the field of immunoassays, especially when both components are known. In addition, including a filtering device to remove certain components is also standard in the art and as such not suitable to confer an inventive activity.

Claim 35 thus does not meet the requirements of Article 33(3) PCT.

4.2.2 Claims 36-42 do not contain any features which, in combination with the features of claim 35 to which they refer, meet the requirements of the PCT in respect of inventive step, the reasons being as follows:

- (i) Using a piezoelectric-based system for the detection of explosives such as TNT is known in the art, as is the use of antibodies directed thereto (claims 36-38) (e.g. document D3, page 4, lines 24-26; page 9, lines 10-17).
- (ii) Choosing the antibody of the present application, designated 5B3 (claim 39), does not seem to be associated with a surprising technical effect and is considered as being an mere selection among a number of known possibilities not involving an inventive activity (see also PCT Guidelines, IV-8.8(C1)(i)) (see also item 3.2.2 above).
- (iii) Removing substances by affinity binding (claim 40) is a standard method in the art.
- (iv) Using an antibody that does not interfere with the biologic activity of a molecule to be detected (claim 41) is obvious for the skilled artisan.
- (v) Claim 42 relates to standard enzymes used for detection in immunoassays.

Therefore, claims 36-42 are not considered as being inventive within the meaning of Article 33(3) PCT.

4.2.3 An analogous argumentation as put forward under items 4.2.1 and 4.2.2 above also applies to the subject-matter of claims 67-73, referring to a method using the system of claims 35-42.

Claims 67-73 therefore do not meet the requirements of Article 33(3) PCT.

5. Invention 5

5.1 Novelty

Claim 77 refers to an antigen-binding protein, having a specificity for TNT. Antibodies selectively binding to TNT are known in the art (see for example document D4, page 14, column 2, line 18; document D5, page 344, lines 44-45; document D6, last paragraph).

Claims 77 and 78 are therefore not novel over the teaching of documents D4-D6 within the meaning of Article 33(2) PCT.

5.2 Inventive step

The subject-matter of claim 74 differs from the teaching of the documents cited under item 5.1 above in that the protein comprises two specific aminoacid sequences.

However, since the use of the antibody of the present application does not seem to be associated with a surprising technical effect it is considered as being an mere selection among a number of known possibilities not involving an inventive activity (see also PCT Guidelines, IV-8.8(C1)(i)).

Claim 74 and dependent claims 75 and 76 therefore do not meet the requirements of Article 33(3) PCT.

Re Item VIII

Certain observations on the international application

1. The term "the explosive" used in point (c) of claim 1 has no antecedent in the preamble (Article 6 PCT).
2. Claims 10, 11, 18, 39, 50, and 70 refer to an antibody which is identified only by a trivial designation which is meaningless to a person skilled in the art. Since said antibody is not clearly identified by its technical features, e.g. aminoacid sequences, said claims do not meet the requirements of Article 6 PCT.

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IL00/00048

3. Claim 35 refers to a sensing member which carries capturing agents for binding to neutralising agents, said neutralising agents being conjugated to an enzyme. However, in figure 33 as well as in example 6, pages 41-43, it is shown that the capturing agent actually bind to the enzyme and not to the neutralising agent (i.e. the antibody). This interpretation is further supported by dependent claim 41, referring to a capturing agent being an antibody which binds the enzyme. The wording of claim 35 is therefore not clear within the meaning of Article 6 PCT.
4. The meaning of the term "substantially" used in claims 74 and 77 is vague and open to interpretation and as such renders the scope of the subject-matter for which protection is sought unclear (Article 6 PCT).
5. Claim 44, being directed to a method, is dependent from claim 32, referring to a system. Due to this inconsistency should claim 44 does not meet the requirements of Article 6 PCT.

INTERNATIONAL SEARCH REPORT

SD

National Application No

T/IL 00/00048

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/53 G01N33/543 C07K16/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

PAJ, EPO-Internal, BIOSIS, WPI Data, CHEM ABS Data, MEDLINE, EMBASE, SCISEARCH, COMPENDEX, STRAND, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 04314 A (YISSUM RES DEV CO ; LEVI SHLOMO (IL); COHEN Yael (IL); DAGAN ARIE () 6 February 1997 (1997-02-06)	1,3-9, 12-15, 17,19, 20,43, 46,47, 49,51,52
Y	page 24, line 3 -page 26, line 26 page 33, line 4 -page 34, line 21 examples 2,6 --- -/--	1,13,14, 35-38, 41-43, 67-70, 72,73



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

° Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document member of the same patent family

Date of the actual completion of the international search

18 October 2000

Date of mailing of the international search report

31 10 2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Nooij, F

INTERNATIONAL SEARCH REPORT

International Application No

/IL 00/00048

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 658 732 A (EBERSOLE RICHARD CALVIN ET AL) 19 August 1997 (1997-08-19) column 5, line 1 - line 35 claims ---	1,13,14, 35-38, 41-43, 67-70, 72,73
X	BRINK S -A: "Bofors Schnauzer-a biosensor for detection of explosives" EUREL INTERNATIONAL CONFERENCE. THE DETECTION OF ABANDONED LAND MINES: A HUMANITARIAN IMPERATIVE SEEKING A TECHNICAL SOLUTION,XX,XX, 7 October 1996 (1996-10-07), pages 33-36, XP002136334 page 34, column 1, paragraph 5 -page 34, column 2, paragraph 2 ---	1-5,9, 12-18, 43-46
X	WO 98 57141 A (SAMUELSSON MAGNUS ;ALM ARNE (SE); ANDERSSON CLAES (SE); ARNELL JOH) 17 December 1998 (1998-12-17) page 4, line 24 -page 6, line 21 ---	1-5,9, 12-18, 43-46
X	EP 0 453 224 A (HUGHES AIRCRAFT CO) 23 October 1991 (1991-10-23) abstract column 2, line 20 - line 31 ---	1-5,9, 12-18, 43-46
A		74-78
A	US 4 999 284 A (WARD MICHAEL D ET AL) 12 March 1991 (1991-03-12) column 2, line 47 -column 3, line 39 ---	1,13,14, 35-43, 67-73
A	PATENT ABSTRACTS OF JAPAN vol. 014, no. 034 (C-679), 23 January 1990 (1990-01-23) & JP 01 269485 A (MATSUSHITA ELECTRIC IND CO LTD), 26 October 1989 (1989-10-26) abstract ---	74-78
A	PATENT ABSTRACTS OF JAPAN vol. 014, no. 034 (C-679), 23 January 1990 (1990-01-23) & JP 01 269488 A (MATSUSHITA ELECTRIC IND CO LTD), 26 October 1989 (1989-10-26) abstract ---	74-78
	--- -/--	

INTERNATIONAL SEARCH REPORT

International Application No

IL 00/00048

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	D. FETTEROLF ET AL.: "An enzyme-linked immunosorbent assay (ELISA) for trinitrotoluene (TNT) residue on hands." JOURNAL OF FORENSIC SCIENCES, vol. 36, no. 2, March 1991 (1991-03), pages 343-349, XP000946955 Philadelphia, PA, USA abstract	74-78
A	U. NARANG ET AL.: "Multianalyte detection using a capillary-based flow immunosensor." ANALYTICAL BIOCHEMISTRY, vol. 255, no. 1, 1 January 1998 (1998-01-01), pages 13-19, XP002150368 abstract figure 1 page 13, right-hand column, line 4 - line 19	74-78



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : G01N 33/48	A2	(11) International Publication Number: WO 00/43774 (43) International Publication Date: 27 July 2000 (27.07.00)
(21) International Application Number: PCT/IL00/00048 (22) International Filing Date: 25 January 2000 (25.01.00) (30) Priority Data: 128212 25 January 1999 (25.01.99) IL (71) Applicants (for all designated States except US): YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM [IL/IL]; Jabotinsky Street 46, 92182 Jerusalem (IL). YEDA RESEARCH AND DEVELOPMENT COMPANY LTD. [IL/IL]; P.O. Box 95, 76100 Rehovot (IL). (72) Inventors; and (75) Inventors/Applicants (for US only): WILLNER, Itamar [IL/IL]; Hashalom Street12, 90805 Mevaseret Zion (IL). ESHHAR, Zelig [IL/IL]; Hess Street18, 76346 Rehovot (IL). (74) Agent: REINHOLD COHN AND PARTNERS; P.O. Box 4060, 61040 Tel Aviv (IL).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: DETECTION OF SMALL MOLECULES BY USE OF A PIEZOELECTRIC SENSOR (57) Abstract Apparatus and method of detecting assayed molecules in a sample make use of a sensing member comprising a piezoelectric crystal with a sensing surface that can interact with a medium in contact therewith by either binding a first indicator agent from the medium, or by releasing a second indicator agent originally immobilized on the sensing surface into the medium. The binding or release causes a change in mass detectable by the use of an electric or electronic utility, through a change in resonance frequency. The medium interacting with the sensing member is a treated sample preparation obtained by reacting the sample with one or both of a reagent solution or sample-processing hardware, such that said medium comprises a first indicator agent or a second indicator agent-releasing species at a concentration of said agent or species which is in correlation to the concentration of the assayed molecules in the sample.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

DETECTION OF SMALL MOLECULES BY USE OF A PIEZOELECTRIC SENSOR

5 FIELD OF THE INVENTION

The present invention is generally in the field of sensors, and concerns a sensing apparatus, system and method for use in detecting of small molecules in a sample.

PRIOR ART

10 In the following description, reference will be made to several prior art documents shown in the lists below. These references will be referred to in the text by indicating the number from this list.

REFERENCES

- 15 1. Shons, A, Dorman, F., Najarian, J., *J. Biomed. Mater. Res.*, **6**:565 (1972).
2. Suleiman, A.A., and Guilbault, G.G., *Analyst*, **119**:2279, (1994).
3. Ward, M.D., and Buttry, D.A., *Science*, **249**:1000, (1990).
4. Oliveria, J.R., and Silver, S.F., U.S. Patent 4,242,096, (1980).
5. Rice, T.K., U.S. Patent 4,236,893, (1980).
- 20 6. Rice, T.K., U.S. Patent 4,314,821, (1982).
7. Roederer, J.E., Bastiaans, G.J., *Anal. Chem.*, **55**:2333, (1983).
8. Roederer, J.E., Bastiaans, G.J., U.S. Patent 4,735,906, (1988).
9. Muramatsu, H., Dicks, J.M., Tamiya, E., and Karube, I., *Anal. Chem.*, **59**:2760, (1987).
- 25 10. Mueller-Schulte, D., and Laurs, H., CA. 112(7), 51807 g, (1990).

11. Muramatsu, H., Kajiware, K., Tamiya, E., and Karube, I., *Anal. Chim. Acta*, **188**:257, (1986).
12. Muramatsu, H., Watanabe, Y., Hikuma, M., Ataka, T., Kubo, I., Tamiya, E. and Karube, I., *Anal. Lett.*, **22**:2155, (1989).
- 5 13. Konig, B., Grätzel, *Anal. Lett.*, **26**:1567, (1993).
14. PCT published application WO 97/04314.

BACKGROUND OF THE INVENTION

The linear relationship between the change in the oscillation frequency of a piezoelectric crystal and the mass variation on the crystal as a result of binding or
10 absorption phenomena, has been used to advantage in a gravimetric monitoring of antigen-antibody binding. The mathematical relationship between the frequency changes of a piezoelectric crystal, Δf , and mass changes, Δm , on the crystal is given by the following Sauerbrey equation:

$$\Delta f = -2.3 \times 10^6 f_0^2 \cdot \Delta m / A$$

15 where f_0 is the fundamental resonance frequency of the crystal prior to the mass variation and A is the surface area of deposited mass. For example, for a crystal exhibiting a fundamental frequency of 9 MHz and surface area of 1 cm², a mass-change on the crystal that corresponds to 1x10⁻⁹ g, will stimulate a frequency change, Δf , of 6 Hz.

20 The first analytical use of piezoelectric crystals in relation to antigen- antibody (Ag-Ab) interactions was reported in 1972⁽¹⁾, where a nyabar precoated crystal was further coated via hydrophobic interactions, with bovine serum albumin (BSA) and the association of the BSA-Ab to the crystal was monitored by the frequency changes. Since then, the piezoelectric detection of antigens and antibodies by piezoelectric
25 means or the quartz crystal microbalance (QCM) has been adopted in a series of analytical studies. The progress in this area has been reviewed by Suleiman *et al.*, 1994⁽²⁾ and Ward *et al.*, 1990⁽³⁾.

Several patents describe the application of QCM for the analysis of antigens and antibodies. Physical adsorption of antigens to a crystal was used as a means for the detection of antigens by interacting the crystal with a mixture of the analyte antigen and a predetermined amount of Ab⁽⁴⁾. The decrease in the antigen concentration was inversely related to the antigen concentration in the sample. In two patents by Rice^(5,6), methods for the determination of Abs by QCM were disclosed. The antigen was immobilized on a polymer precoated crystal and the frequency changes as a result of Ab association related to the analyte Ab concentration in the sample. By this method, human IgG against honey bee venom, phospholipase A, and keyhole limpet hemocyanine were analyzed⁽⁷⁾. However, non-specific binding to the crystal interfered with the analyses. In a follow-up patent⁽⁸⁾, the detection of low molecular weight components by a pre-coated crystal with the anti-Ab and competitive binding assay of the Ab-low molecular weight analyte was described. All of these analyses were performed by treatment of the crystals in solution and subsequent frequency measurements in air. This two-step solution/gas procedure allows improvement of the sensitivity of the resonating QCM, but introduces technical complications and the interference of hydration/dehydration phenomena that are reflected in the frequency parameters.

Piezoelectric immunoassaying in the liquid phase has important technical advantages as it allows stationary and flow analysis of aqueous samples. The method suffers, however, from a basic physical limitation due to substantially lower frequency changes of the crystal as a result of the solution viscosity. QCM immunoassays in solution were reported by Roederer⁽⁷⁾ and addressed in a follow-up patent⁽⁸⁾. The quartz crystal was modified with glycidoxypyltrimethoxy silane (GOPS), and the surface-modified crystal was then further modified by anti-human IgG antibody and then applied for the piezoelectric detection of human IgG. The detection limit of the device was determined to be $13 \mu\text{g}\cdot\text{ml}^{-1}$. A closely related approach was adopted by Muramatsu *et al.*⁽⁹⁾ where the quartz crystals were surface-modified by γ -aminopropyl triethoxy silane and further derivatized by protein A. The surface-modified crystals were then applied for the determination of human IgG in the concentration range

10^{-6} - 10^{-2} mg·ml⁻¹. A related patent disclosed the piezoelectric analysis of thyroxine using a polyamide 6 polymer coating and anti-thyroxine Ab as sensing interface⁽¹⁰⁾.

Piezoelectric analysis of high molecular weight antigens such as microbial cells was addressed using antibody-coated quartz - crystals. *C.albicans* cells in the concentration range 1×10^6 - 5×10^8 cells·ml⁻¹ were analyzed by an anti-*Candida albicans* Ab surface⁽¹¹⁾, *E.coli* with an anti-*E.coli* interface⁽¹²⁾ and protein A-coated crystals acted as piezoelectric sensing interface for various bacteria including *Salmonella*, *Shigella*, *Yersinia* and *E.coli*⁽¹³⁾. Various schemes allowing efficient use of QCM in assaying analytes in a liquid media have been described in WO 97/04314⁽¹⁴⁾.

QCM techniques were used hitherto only for detection of interaction with substances of relatively high molecular weight, e.g. proteins and other macromolecules. The QCM techniques are generally considered not sufficiently sensitive for detection of substances of a low molecular weight.

GENERAL DESCRIPTION OF THE INVENTION

The present invention has as its object the provision of an apparatus, system and method for detecting small assayed molecules in a sample.

The term “*detection*” as used herein means to denote either or both of a qualitative determination of the existence of an explosive in a sample or a quantitative analysis of the amount or concentration of such an explosive in the sample.

The term “*sample*” means to denote a liquid preparation which may either be a specimen in which an explosive is to be detected, may be a fractionation product which contains the explosive if present in a sample, or in general any preparation in which the level of explosive is indicative to the level of explosive in the specimen. The specimens which may be tested in accordance with the invention may be an original liquid specimen, e.g. samples withdrawn from water reservoirs, may be a soil specimens or gas specimens. In the case of gasses or soil specimens, these are typically first reacted with the liquid in a manner that at least some of the explosive molecules if present in the specimen will be dissolved in the liquid, typically an aqueous solution.

The term "*small molecule*" as used herein means to denote a molecule having a molecular weight such that its binding to a sensing surface at an amount expected to be found in a sample will not give rise to a detectable mass change of the sensing surface. In particular, the term "*small molecules*" as used herein applies
5 to molecules having a molecular weight below about 5,000 Dalton, typically below about 1,500 Dalton, at times even below about 500 Dalton. Specific examples of small molecules may be detected in accordance with the invention are explosive molecules including, but not limited to, DNT and TNT.

Explosives have a relatively low volatility and accordingly, a specimen even if
10 taken from the vicinity of an explosive, typically contains only a small amount of such molecules. Thus, any method intended for sensing the presence of explosive molecules in a sample should be highly sensitive and adapted for detecting a small amount of explosive molecules. This may be the case also with respect to other types of low molecular weight molecules which may be assayed, such as drugs, e.g. heroin,
15 cocaine, etc.

The present invention provides, by a first of its aspects, an apparatus for detecting small assayed molecules in a sample, comprising:

- (a) a sensing member comprising a piezoelectric crystal having at least one sensing surface which can interact with a medium in contact therewith a by
20 either binding a first indicator agent from the medium, or by releasing a second indicator agent originally immobilized on the sensing surface into the medium; the medium being either the sample in which case the assayed molecule present, causes the release of the second indicator agent from the at least one sensing surface, or being a treated sample preparation obtained by
25 reacting the sample with one or both of a reagent solution or sample-processing hardware, such that said medium comprises a first indicator agent or a second indicator agent-releasing species at a concentration of said agent or species which is in correlation to the concentration of the assayed molecule in the sample, the binding or release
30 resulting in a change of mass of the sensing surface;

- (b) a testing cell for holding said medium and bringing it into contact with said at least one sensing surface; and
- (c) an electric or electronic utility for inducing vibrations in the piezoelectric crystal and measuring resonance frequency thereof, a change in resonance frequency after contact between the sensing surface and the medium, indicating presence of the explosive in the sample.

The invention also provides a method for detecting a small molecule in a sample, comprising:

- (a) providing a sensing member comprising a piezoelectric crystal having at least one sensing surface which can interact with a medium in contact therewith by either binding a first indicator agent from the medium, or by releasing a second indicator agent originally immobilized on the sensing surface into the medium;
- (b) contacting the at least one sensing surface with a medium being either the sample in which case the assayed molecule if present causes the release of the second indicator agent from the sensing surface, or being a treated sample preparation obtained by reacting the sample with one or both of a reagent solution or sample-processing hardware, such that said medium comprises a first indicator agent or a second indicator agent-releasing species, at a concentration of said agent or species which is in correlation to the concentration of the assayed molecule in the sample, the binding or release resulting in a change of mass of the sensing surface;
- (c) inducing vibrations in the piezoelectric crystal and measuring resonance frequency thereof; and
- (d) determining whether a change in resonance frequency after contact between the sensing surface and the medium occurred, such change indicating presence of the assayed molecule in the sample.

In accordance with a preferred embodiment of the invention the small molecule is an explosive. Particular, non-limiting examples of explosives are dinitrotoluene (DNT) and 2,4,6-trinitrotoluene (TNT).

The apparatus of the invention may be designed to provide an all-or-none response, namely an indication whether the assayed small molecule is present in the sample. Alternatively, the apparatus may also be used and designed to provide a quantitative response, namely a response that depends on the level of the assayed molecule in the sample.

In accordance with one embodiment of the invention, said at least one sensing surface carries capturing agents which bind to neutralizing agents at an assayed molecule-binding domain thereof. In accordance with this embodiment, the sensing member is contacted with a medium which contains capturing agents at a concentration which inversely reflects the concentration of the assayed molecules in the sample. In accordance with this embodiment, the sample is first contacted with a reagent solution comprising the neutralizing agents which then bind the assayed molecules if such are in the sample. This treated preparation is then brought into contact with the at least one sensing surface. The neutralizing agents, bound to the assayed molecules, are not any more free to bind to the capturing agents. Thus, where the sample is free from the assayed molecules, a relatively large number of neutralizing agents will be free to bind to the capturing agents on the at least one sensing surface. Where the sample contained the assayed molecules, there will be less neutralizing agents free to bind to the capturing agents. Thus, a decrease in the resonance frequency, indicative of increased immobilized mass on a surface, is a reverse indication of the presence of the assayed molecule in the sample: a relatively large decrease in resonance frequency indicates absence or little concentration of the assayed molecules in the sample, and no or only a little decrease indicates existence of the assayed molecule in the sample. The extent of decrease in resonance frequency versus control can be used as inverse indication of the concentration of the assayed molecule in the sample. The capturing agents are typically residues or moieties of the assayed molecules and the neutralizing agents may comprise first antibodies which have a relatively high specific binding affinity to the assayed molecules.

The term "*antibody*" as used herein is meant to encompass antibodies of various kinds including, but not limited to IgG or IgM antibodies which may be polyclonal or monoclonal (mAb). In addition, the term "*antibody*" encompasses also various derivatives of antibodies which include the antigen-binding domain of the antibody. Such derivatives may include Fc-less antibodies (antibodies devoid of the constant region), single chain antibodies, constructs comprising only the variable region of the antibodies, etc.

In accordance with another embodiment of the invention, the at least one sensing surface carries residues or moieties of the assayed molecules bound to first antibodies, which competitively bind to soluble assayed molecules in a medium which comes into contact with the at least one sensing surface. In the presence of the assayed molecule in said medium the antibodies are released from the at least one sensing surface to bind the assayed molecules. An assay where an apparatus in accordance with this embodiment is used, involves an essential competition reaction between the assayed molecule and the capturing agent on binding to the neutralizing agent. In accordance with this embodiment the sample is brought into direct contact with the at least one sensing surface. In the presence of the assayed molecule in the sample, neutralizing agents are released from the sensing surface to bind to the assayed molecules in this surrounding solution. Thus, an increase in the resonance frequency, indicative of a reduction in mass immobilized on the at least one sensing surface, only indicate the presence of the assayed molecule in the sample. Against this, lack of change or little change in the resonance frequency indicates no or only a small amount of the assayed molecule in the sample.

The first antibodies in both of the above embodiments may be bound or complexed with a mass-increasing agent for the purpose of amplifying the response. A mass-increasing agent may, for example, be a large molecular complex, e.g. a protein, conjugated to the antibody, or a colloid particle, e.g. a gold colloid, bound to the first antibody. Such a second antibody may be conjugated to or complexed with other agents such as to a colloid particle, to a molecular complex, etc. In addition, the mass-increasing agent may, by one preferred embodiment, comprise a

second antibody which binds to said first antibody. In addition, by another preferred embodiment, the mass-increasing agent, which may be any of the agents mentioned above, comprises avidin or streptavidin which then binds to a biotin residue conjugated to the first antibody. The mass-increasing agent may be added either
5 prior to allowing the first antibodies to bind to the at least one sensing surface or thereafter. Where a mass-increasing agent is used, the change of mass as a result of binding to release of the neutralizing agent will be considerably larger, and consequently there will be a larger signal-to-noise ratio.

As stated above, the assayed molecule is typically an explosive molecule such
10 as DNT or TNT. In such a case said first antibodies, which are typically mAbs, are preferably mAbs with the binding characteristics of 5B3, particularly in the 5B3 mAb itself.

The present invention further provides a system for detecting small assayed molecules in a sample which comprises the above apparatus as well as one or both
15 of reagents and hardware for processing the sample or for introducing it into the assayed cell of the apparatus. The system typically has hardware components for propelling the sample into the assayed cell. The system may have various additional hardware and reagents which are intended for processing of the sample, for contacting the sample with reagents, etc.

20 In accordance with one embodiment, to be referred to herein as the "*displacement embodiment*" the system comprises:

- (a) a sensing member comprising a piezoelectric crystal having at least one sensing surface which carries residues or moieties of said assayed molecules bound to neutralizing agents, e.g. anti-assayed molecule antibodies;
- 25 (b) a testing cell for holding a medium and bringing it into contact with said at least one sensing surface, whereby in the presence of the assayed molecules in the medium, at least some of said antibodies are released into the medium;
- (c) hardware for introducing the sample into the testing vessel; and
- (d) an electric or electronic utility for inducing vibrations in the piezoelectric
30 crystal and measuring resonance frequency thereof, a reduction in resonance

frequency after contact between the sensing surface and the medium, indicating presence of said assayed molecule in the sample.

In accordance with the displacement embodiment the sample is contacted with the sensing surface and then vibrations are induced in the piezoelectric crystal to measure resonance frequency. Owing to binding competition, where the sample comprises assayed molecules, some of the neutralizing agents are released and bind to the assayed molecules in the sample which results in reduction of mass of the sensing surface and thus an increase in the resonance frequency. Such an increase is then indicative that the assayed molecule is present in the sample.

10 In accordance with another embodiment, to be referred to herein as the "*competition embodiment*", the system comprises:

- (a) a sensing member comprising a piezoelectric crystal having at least one sensing surface which carries residues or moieties of the assayed molecules;
- (b) a reagent system comprising neutralizing agents, e.g. anti-assayed molecule antibodies;
- 15 (c) a testing cell for holding a medium and bringing it into contact with said at least one sensing surface, whereby in the presence of the assayed molecules in the medium, at least some of said antibodies are released into the medium;
- (d) an arrangement for contacting the sample with said reagent system to obtain a treated sample preparation and for introducing the treated sample preparation into the testing vessel; and
- 20 (e) an electric or electronic utility for inducing vibrations in the piezoelectric crystal and measuring resonance frequency thereof, a decrease in resonance frequency after contact between the sensing surface and the treated sample preparation, indicating that the sample is free of the assayed molecules.

25 In accordance with the competition embodiment the sample is contacted with neutralizing agents and incubated for a time allowing the neutralizing agents to bind the assayed molecules if such are present in the sample. This treated sample preparation is then contacted with the at least one sensing surface. In the presence of the assayed molecules in the sample, the neutralizing agents will bind these

30

molecules and will thus not be free to bind to the capturing agents. Thus, in the presence of the assayed molecules in the sample, there will be no or little change in the resonance frequency of the crystal. If the sample is free of the assayed molecule, the neutralizing agents will be free to bind to the capturing agents which will give
5 rise to a significant mass increase and hence to a decrease in the crystal's resonance frequency. Thus, such a decrease will indicate absence of the assayed molecules from the sample.

In accordance with another embodiment, to be referred to herein as the "*basic filtration embodiment*", the system comprises:

- 10 (a) a sensing member comprising a piezoelectric crystal having at least one sensing surface which carries capturing agents for binding to neutralizing agents;
- (b) a testing cell for holding a medium and bringing it into contact with said at least one sensing surface;
- 15 (c) a reagent system comprising the neutralizing agents which can bind to the assayed molecules;
- (d) an arrangement for contacting the sample with the reagent system under condition and for a time permitting binding of the neutralizing agent to the assayed molecules, to obtain a treated sample preparation;
- 20 (e) a filtration system for filtering out from said treated sample preparation neutralizing agents unbound to an explosive molecule to obtain a filtrate essentially devoid of such unbound neutralizing agents;
- (f) arrangement for transfer of said filtrate to said testing cell; and
- (g) an electric or electronic utility for inducing vibrations in the piezoelectric
25 crystal and measuring resonance frequency thereof, an increase in resonance frequency after contact between the sensing surface and the filtrate, indicating presence of the assayed molecules in the sample.

In accordance with the basic filtration embodiment, the sample is contacted with a neutralizing agent, typically an antibody, which can bind to the assayed
30 molecules. The incubation of the sample with the neutralizing agent is contacted under

conditions and for a time permitting binding of the neutralizing agents to the assayed molecules thus obtaining a treated sample preparation. The treated sample preparation is then filtered through the filtration system to filter out neutralizing agents which are unbound to the assayed molecules so as to obtain a filtrate essentially devoid of such unbound neutralizing agents. The filtrate is then contacted with the at least one sensing surface. Where the sample contained the assayed molecules, the neutralizing agents will filter through the filtration system and will eventually bind to the at least one sensing surface resulting in a mass increase. In the absence of the assayed molecules from the sample, the neutralizing agents will be captured within the filtration system and contact of the filtrate with the sensing surface will not give rise to an increase in mass. Thus, a decrease in resonance frequency, indicative of binding of neutralizing agents to the at least one sensing surface, will signify presence of the assayed molecules in the sample.

In accordance with yet another embodiment of the invention, to be referred to herein as the "*enzyme embodiment*", the system comprises:

- (a) a sensing member comprising a piezoelectric crystal having at least one sensing surface which carries capturing agents for binding to neutralizing agents;
- (b) a testing cell for holding a medium and bringing it into contact with said at least one sensing surface;
- (c) a reagent system comprising neutralizing agents which can bind to the assayed molecules, said neutralizing agent being conjugated to an enzyme which can catalyze a reaction yielding an insoluble reaction product;
- (d) an arrangement for contacting the sample with the reagent system under condition and for a time permitting binding of the neutralizing agent to the assayed molecules, to obtain a treated sample preparation;
- (e) a filtration system for filtering out from said treated sample preparation neutralizing agents unbound to an assayed molecule to obtain a filtrate essentially devoid of such unbound neutralizing agents;
- (f) arrangement for transfer of said filtrate to said testing cell;

- (g) an ensemble of reagents and conditions for inducing said enzyme to catalyze the reaction yielding the insoluble reaction product; and
- (h) an electric or electronic utility for inducing vibrations in the piezoelectric crystal and measuring resonance frequency thereof, an increase in resonance frequency after contact between the sensing surface and the filtrate or after
5 permitting the enzyme to catalyze said reaction, indicating presence of the assayed molecule in the sample.

This embodiment is somewhat similar to that previously described, the difference is that there is an additional amplification sequence in which the enzyme is
10 permitted to catalyze a reaction giving rise to unsoluble reaction products which in the presence of the assayed molecule in the sample, give a higher increase in mass and hence a higher decrease in resonance frequency.

The invention also provides, by another of its aspects, a monoclonal antibody designated herein as 5B3. The nucleotide sequence and the deduced amino acid
15 sequence of the heavy and light chain variable regions of the 5B3 monoclonal antibody are depicted in Figs. 3A and 3B, respectively. An antigen-binding portion of an antibody can be fused (chemically or by creating a fused gene containing the coding sequence and expressing such a sequence) in a manner such that said antigen-binding portion in the resulting fused protein retains its antigen-binding
20 specificity. Thus, the present invention encompasses in its scope any protein, particularly an antibody, which includes an antigen-binding portion with substantially the same antigen-binding specificity of the 5B3 antibody and having two cooperating peptide sequences being the sequences of Fig. 3A and Fig. 3B, or of altered sequence thereof, as defined below. Such an antibody may be of any known type, e.g. IgM or
25 IgG, may be a fragment of an antibody comprising the antigen-binding domain, e.g. Fab, an antibody without the Fc region, a single chain antibody, etc.

As will readily be appreciated by the artisan it is possible, at times, to alter the variable antigen-binding portion, to obtain an altered portion, by adding one or more amino acid residues, deleting one or more amino acid residues or replacing one or
30 more amino acid residues by other residues, such that the altered portion retains

substantially the same antigen-binding specificity of the non-altered portion, i.e. that of the 5B3 antibody. Thus, such altered variable regions as well as antibodies or other constructs including such altered variable regions, with the altered variable region retaining substantially the same antigen-binding specificity of the non-altered variable region (namely that depicted in Figs. 3A and 3B) are also included within the scope of the present invention.

The term "*retaining substantially the same antigen-binding specificity*" should be understood as meaning that the altered portion has the ability to bind an antigen (e.g. TNT or DNT) bound by the non-altered portion with an affinity which is considerably higher (typically by several orders or magnitude) than the affinity of binding to other antigens. Such altered portion may have a binding affinity to the antigen which may be about the same as that of the non-altered portion or with the binding affinity which may at times be somewhat higher or somewhat lower than that of the non-altered portion.

The at least one sensing surface typically carries a metal plate made particularly of such metals having the capability to associate chemical with, attach or chemisorb a sulfur-containing moiety. The metal plates are preferably made of or coated by metals such as gold, platinum, silver or copper. Where the capturing agents are carried on metal plates these typically thus serve as a dual role of both serving as a carrying matrix for the capturing agent as well as being an electrode of the piezoelectric crystal device.

The capturing agents are typically immobilized to the metal plates by means of a sulfur containing moiety bound thereto, e.g. in the manner described in WO 97/04314⁽¹⁴⁾.

The term "*neutralizing agent*" which is used herein means to denote an agent which has a specific binding affinity to the assayed molecules. In accordance with one embodiment the neutralizing agent is an antibody with a specific binding affinity to the assayed molecule. The embodiment in which the neutralizing agent is an antibody, typically a monoclonal antibody (mAb) is a preferred embodiment of the invention. However, the term "*neutralizing agent*" encompasses also other types of agents

including, for example: a soluble receptor moiety containing the analyte binding domain, in which case the assayed molecule is said analyte; a soluble receptor moiety containing the binding domain of the enzymes' substrate wherein the assayed molecule is said substrate; and others. In addition, the neutralizing agent may also be a synthetic macromolecule which comprises one domain with binding affinity to the
5 assayed molecule, e.g. a binding domain derived from an anti-assayed molecule antibody, conjugated or fused to a macromolecular moiety, e.g. a large polypeptide or protein. (Conjugation or fusion may be achieved, as known, by chemical binding, by genetic engineering techniques, etc.).

10 The invention will now be further illustrated in the following detailed description of some preferred embodiments, with reference made to the annexed drawings. As will be appreciated, this description is for the purpose of illustration only and is not intended to be limiting.

15 BRIEF DESCRIPTION OF THE DRAWINGS:

Fig. 1A and Fig. 1B show the structure of the various haptens, being TNT derivatives (Fig. 1A) or DNT derivatives (Fig. 1B), which were used in the embodiments described herein. In the Figs. the abbreviation P denotes KLA, F γ G or BSA, the abbreviation TNP-cap denotes TNP-caproic acid and the abbreviation
20 2,4-DNP-cap denotes 2,4-DNP-caproic acid.

Fig. 2A shows inhibition profiles of binding (performed by ELISA, I denotes Inhibition and B denotes Binding) of 5B3 antibody to DNS-BSA (DNS coupled to BSA) in the presence of different inhibitors – TNT, TNP-caproic acid (TNP-cap), DNP-caproic acid (DNP-cap), mDNB, 2,4-DNA, DNS, 2,6-DNT and 2,4-DNP.

25 Fig. 2B shows inhibition profiles (performed by ELISA, I denotes Inhibition and B denotes Binding) of binding of 5B3 antibody to TNP₂-F γ G (TNP coupled to F γ G) in the presence of different inhibitors – TNT, TNP-caproic acid (TNP-cap), DNP-caproic acid (DNP-cap), mDNB, 2,4-DNA, DNS, 2,6-DNT and 2,4-DNP.

Fig. 3A and Fig. 3B show, respectively, the nucleotide sequences (SEQ ID Nos. 5 and 7) and the deduced amino acid sequences (SEQ ID Nos. 6 and 8) of the 5B3 heavy and light chain variable regions.

Fig. 4 shows a chemical scheme of production of a sensing surface containing a monolayer of capturing agents. The abbreviations **EDC**, **NHS**, **CYST** and **3,5-DNSA**, denote, respectively, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, N-hydroxysulfosuccinimide, cystamine and 3,5-dinitro-salicylic acid.

Fig. 5 shows the frequency changes (**T/min** denotes Time in minutes) of the crystal during its incubation with the cystamine and the formation of a system in a monolayer.

Fig. 6 shows the frequency changes (**T/min** denotes Time in minutes) of the crystal during the process of binding of the capturing agent to the system in monolayer.

Fig. 7 is a schematic illustration of a basic configuration of the displacement embodiment.

Fig. 8 is a schematic, more detailed illustration of a measuring cell with the sensing measure in accordance with an embodiment of the invention.

Fig. 9 shows the resonance frequency decrease (**T** denotes Time in seconds) of the crystal upon association of the anti-DNP antibodies ("*DNP-Ab*") to the sensing surface.

Fig. 10 shows the crystal frequency changes (**T/min** denotes Time in seconds) during incubation of the DNP-Ab containing layer with a medium comprising 2,4-DNT.

Fig. 11 shows the decrease in the crystal's resonance frequency (**T/sec** denotes Time in seconds) upon binding of the mAb 5B3 to a cystamine/3,5-dinitrosalicylic acid monolayer.

Fig. 12 shows the increase in frequency (**T/sec** denotes Time in seconds) of the crystal of Fig. 11 upon injection of 2,4-DNT to the cell at a concentration of 6.25 ng/ml (curves A and B) and 312 pgr/ml (curve C).

Fig. 13 shows the frequency increase (T/sec denotes Time in seconds) upon injection of TNT at a concentration of 6.25 pgr/ml (curve A) and 62.5 pgr/ml (curve B), of the sensing member of the kind used in Fig. 12.

Fig. 14 shows the resonance frequency decrease (T/sec denotes Time in seconds) of the electrode employed in Fig. 13, upon recharging the monolayer with the 5B3 antibody.

Fig. 15 shows the frequency increase (T/sec denotes Time in seconds) of the electrode of Fig. 14, upon contact with a sample containing 6.25 pgr/ml TNT.

Fig. 16 is a schematic illustration of a configuration of the displacement embodiment wherein a mass-increasing agent is used.

Fig. 17 shows the decrease in resonance frequency of a crystal (T/sec denotes Time in seconds), upon addition of biotinylated antibody to the antigen monolayer on the sensing surface.

Fig. 18 shows the time-dependent frequency decrease (T/sec denotes Time in seconds) of a crystal charged with biotinylated antibodies, upon interaction with avidin.

Fig. 19 shows the crystal resonance frequency increase (T/sec denotes Time in seconds) upon exposure of a sensing surface containing a DNT-antibody-avidin layer to a 2,4-DNT sample.

Fig. 20 is a general, schematic illustration of a system in accordance with an embodiment of the invention.

Fig. 21 shows the frequency changes (T/sec denotes Time in seconds) upon interaction of a capturing agent-comprising sensing surface with specific antibodies, in a system of the kind shown in Fig. 20.

Fig. 22 shows the change in frequency of the crystal shown in Fig. 21 (T/sec denotes Time in seconds), upon exposure to a TNT-containing sample.

Fig. 23 is a schematic illustration of a basic configuration of the competition embodiment of the invention, in the absence (-Ag) or presence (+Ag) of antigen.

Fig. 24 illustrates a configuration of the competition embodiment, wherein use is made of a mass-increasing agent, in the absence (-Ag) or presence (+Ag) of antigen.

Fig. 25 shows the results of an assay performed in accordance with the competition embodiment (**T/min** denotes Time in minutes), upon exposure of the sensing surface to a reference system lacking dinitrophenol (curve a), to a DNP-Ab/anti-Ab probe solution lacking the DNP antigen (curve b) and upon exposure to the DNP-Ab/anti-Ab probe solution including 3.1×10^{-12} g of DNT (curve c).

Fig. 26 shows the frequency changes in a crystal of the kind of Fig. 25 (**T/min** denotes Time in minutes) exposed to DNP-Ab/anti-Ab probe solution mixed with 2,4-DNT (curves a and b) and exposure to a probe solution without 2,4-DNT (curve c).

Figs. 27A-27C show frequency changes of a crystal (in an assay performed in accordance with the competition embodiment, **T/sec** denotes Time in seconds) upon exposure to different DNT-isomer positive (curve b in the three figures) and in the presence of non-DNT contaminated samples (curves a in the three figures), the isomers being 2,4-DNT in Fig. 27A, 1,4-DNT in Fig. 27B and 2,6-DNT in Fig. 27C.

Fig. 28 shows the frequency changes (**T/sec** denotes Time in seconds) of different electrodes which have been stored for 7, 15 and 28 days (Fig. 28A, Fig. 28B and Fig. 28C, respectively), in an assay performed in accordance with the displacement embodiment.

Fig. 29 is a schematic illustrating of the basic filtration embodiment, in the absence (-Ag) or presence (+Ag) or antigen.

Fig. 30 outlines a step-wise modification of a gold-sensing surface associated with the quartz crystal to obtain a biotin-avidin layer (**BNHSE** denotes Biotinamidocaproate N-hydroxysuccinimide ester).

Fig. 31 shows the crystal resonance frequency changes (**T/sec** denotes Time in seconds) as a result of charging a biotin monolayer with avidin.

Fig. 32 shows a change in the resonance frequency (**T/sec** denotes Time in seconds) of the crystal of Fig. 31 upon exposure of the sensing surface to a reference system (curve a, no analyte) in the case of a sample containing 2,4-DNT (curve b).

Figs. 33 and 34 are schematic illustrations of the enzyme embodiment (**DNT/TNT-SAMP** denoting a sample containing DNT and TNT, **C-SAMP** denoting a clean sample, **SUB** denoting substrate and **PROD** denoting product).

5 DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The following are some exemplary embodiments of the invention:

1. Anti-TNT antibodies

1.1 Antigens/Immunogens

10 The following TNP-proteins have been used: TNP₄₀-KLH, TNP₃₃-BSA, TNP₂-F γ G. To simulate the antigen subsequently used on the QCM crystal (see below), 2,4-dinitrosalicylic acid (DNS) coupled to BSA was used. Coupling between the TNT, DNT and derivatives and the protein was done using EDCI. The structure of TNT and derivatives as well as that of DNT and derivatives which were used is
15 shown in Figs. 1A and 1B.

As immunogen, 2,4,6-trinitrophenyl (TNP) was used which after coupling to a protein carrier looks very similar to TNT. TNP was coupled to a carrier protein (keyhole limpet hemocyanine (KLH) as immunogen or bovine serum albumin (BSA) and fowl gamma globulin (F γ G) as antigen), by mixing 100 mg 2,4,6-trinitrobenzene
20 sulfonic acid (TNBS) with 100 mg protein at 0.2 M borate buffer pH 9.2 for 2 hours at 37°C. Following extensive dialysis against PBS, the TNP-proteins were filtered through a 45 μ m filter and the molar ratio of TNP per protein was determined using molar extinction coefficient of 15,400 at 348 nm⁽¹⁾.

25 1.2 Immunization

Female BALB/c mice 8-10 weeks of age were immunized intradermally into the hind foot pads with 20 μ g of TNP-KLH emulsified with Complete Freund's Adjuvant (CFA). Two weeks later, mice were injected subcutaneously with the same amount of antigen in CFA. A week following this boost, mice were bled and the
30 anti-TNP antibody titre was determined in their sera using ELISA (see below). One

month after the second boost, mice showing the highest antibody titre were boosted again intraperitoneally with 10 µg TNP-KLH in PBS on days -4 and -3 prior the cell fusion.

5 1.3 Preparation of 5B3 Monoclonal Antibody

Spleen cells of a mouse with the highest titre of binding antibodies (and specificity to TNT, see for detection details below) were fused with the NSO, murine myeloma cell line as described before (Eshhar, Monoclonal antibody strategy and techniques. In: *Hybridoma in Biotechnology and Medicine*, Springer, T., ed. Plenum Press, p. 1, 1985). Briefly, 10⁸ spleen cells were fused with 2x10⁷ NSO myeloma cells using 41% PEG for 2 mins. at 37°C. Following removal of PEG by dilution and centrifugation, cells were resuspended in DMEM supplemented with 10% horse serum and HAT selective medium and distributed into 7 microculture plates. After 12 days incubation at 37°C in 10% CO₂ saturated air, 50 µl aliquots of the hybridoma culture supernatants were removed in duplicate, one aliquot was assayed for binding to TNP-BSA, the second was assayed similarly, but following preincubation in the presence of TNT (10⁻⁵M). (See binding and inhibition assays (1.4 and 1.5 below). From 672 wells with hybridoma growth 32 scored positive for TNP-BSA binding. Six out of these were inhibited by 10⁻⁵M TNT, out of which a hybridoma designated 5B3 was selected. The TNT-specific hybridomas were cloned and subcloned for 2-3 cycles and used to prepare ascites fluid which served as the source for purified antibodies.

For purification, the immunoglobulin fraction was precipitated from ascites fluids in 45% saturated ammonium sulfate, dialyzed against PBS and loaded on Protein-G-Agarose (Pharmacia) column. Monoclonal antibodies were eluted at pH 2.7, and dialyzed against PBS. Purified antibody preparations were stored in -70°C. The purified antibodies contained more than 96% active antibodies as verified by their ability to bind to antigen- columns.

1.4 Binding Assay for Anti-TNT Antibodies

Regular ELISA has been used to determine anti-TNT antibody activity. Microtitre plates (Maxisorb, NUNC) were coated with antigen (TNP-BSA, TNP-F γ G, DNS-BSA) by incubating them with 100 μ l of 2-10 μ g/ml antigen in PBS for at least
5 two hours in room temperature (RT). Following removal of antigen, plates were blocked by incubation for an hour at RT with PBS supplemented with 0.5% BSA and then washed with PBS supplemented with 0.05% Tween-20 (PBS-Tween). Supernatants or sera containing anti-TNP antibodies serially diluted in PBS supplemented with 0.01% PBS (100 μ l) were then added to the washed plates and
10 after 1 hour incubation at 37°C, antibodies were removed, plates were washed 3 times with PBS-Tween and 100 μ l peroxidase-labeled anti-mouse Fab antibodies (Jackson Labs.), diluted according to the manufacturer's instructions were added. Plates were incubated for an hour at RT, washed (x3) and a peroxidase substrate (2,2'azinodi-(ethylbenzthiazoline sulfonic acid) diammonium salt (ATBS, Sigma) 1
15 mg/ml with 0.003% H₂O₂ in citrate-phosphate buffer, pH 4.5) was added. Reaction was stopped when sufficient color had been developed by the addition of 50 μ l of 0.2 M citric acid and the OD at 620 nm was determined in ELISA plate reader.

1.5 Inhibition Assay

20 To determine the specificity and affinity of the antibodies (from immune sera, supernatants, ascites fluids or purified), antibody dilutions giving approx. 70% of the maximal binding were incubated with different concentrations of the hapten or hapten analogs for 30-60 mins. at RT. Mixtures of antibodies and the inhibitor (the hapten or the hapten analogs) were then transferred to ELISA plate coated with an antigen
25 (DNS-BSA or TNP₂-F γ G) and the residual binding capacity was determined. The results are shown in Fig. 2A and 2B. Degree of inhibition was calculated from % binding of uninhibited sample minus background control. As a practical measure of affinity, the concentration of hapten which gave 50% inhibition, (IC₅₀), was determined.

1.6 Characteristics of 5B3 Antibody

5B3, is an IgG1 antibody, derived from a TNP-KLH immunized mouse. Some of its binding characteristics are summarized in the following Table 1, showing the IC₅₀ of various haptens of binding of the 5B3 antibody to TNP-antigen.

5 TNP-antigen. 5B3 mAb has two important additional features, which can also be seen in Table 1, which render it suitable for use in accordance with the invention: it does not bind avidly to 2,4-DNS which is used, by some embodiments, to coat the QCM crystal, and its binding to either TNP or DNS antigen can be blocked by very low amounts of TNT. These features were indeed found to be very useful since a
10 concentration as low as 6 pgr of TNT could displace bound 5B3 from the QCM crystal and give a reproducible signal.

1.7 DNA and Protein Sequence of 5B3 Antibody

cDNA was prepared from total RNA extracted from the 5B3 hybridoma using
15 reverse transcriptase and oligo dT as 3' primer (Promega kit and protocol book for Reverse Transcription (RT)). To PCR amplify the cDNA coding for the variable region of the light chain, N5'VK2 as 5' and N3'VK as 3' oligonucleotide primers were used. For the amplification of the cDNA coding for the variable region of the heavy chain, N5'VH as 5' and N3'VH as 3' oligonucleotide primers were used. These
20 oligonucleotide primers had the following sequences (SEQ ID Nos. 1 to 4, respectively):

N5'VK2 5'CCCGTCTAGAGGAGAYATYGTWATGACCCAGTCTCCA

NV'VK 5'GTTTKATCTCGAGCTTKGTSCC

N5'VH 5'AGGTSMARCTKCTCGAG

25 N3'VH 5'TGMRGAGACGGTGACCGTRGTYCCTTGGCCCCAG

In the above primer sequences the following letters have the following meanings:

R = A or G K = G or T

S = C or G W = A or T

30 Y = C or T M = A or C

The first and the fourth of the above primers have already been described (Eshhur *et al.*, *PNAS*, **90**:720 (1993)).

PCR amplification was performed as previously described (The polymerase chain reaction, in: *Current Protocols in Molecular Biology*, Vol. 2, Chapter 15, John Wiley & Sons Inc., 1995).

Nucleotide sequencing was carried out directly from the PCR mixtures, reading both strands and using the primers described above. Automated sequencing using the Taq Dye Deoxy Terminator cycle sequencing kit and the ABI PRISM™ 377 DNA Sequencer was carried out. The nucleotide sequence and the deduced amino acid sequences of the 5B3 heavy and light chain variable regions are shown in Fig. 3A and Fig. 3B, respectively.

2. Preparation of a layer of capturing agents on a QCM crystal

In all the experiments reported herein, quartz crystals (AT-cut) sandwiched between two gold (Au) electrodes were used, which had a geometrical area of about 0.2 cm², with a roughness factor which varied between 1.8 and 15. The crystal had a resonance frequency of around 9 MHz.

The chemical scheme of forming the electrodes can be seen in Fig. 4. The electrodes 102, carried on crystal 100 were cleaned by incubation in distilled water for 15 mins. which was following by rinsing three times with water and drying under a stream of nitrogen. The electrodes were then immersed in a 0.2-0.5 M cystamine dihydrochloride 104 solution for 2 hours, then introduced into distilled water for 15 mins. and dried with nitrogen. This gave rise to formation of a cystamine monolayer 106 on electrode 102. This gave rise to a resonance frequency change (Δf) within the range of $\Delta f = (-200) - (-500)$ Hz, as can be seen in Fig. 5, which shows the Δf change with time of incubation in cystamine.

2-5-Dinitrosalicylic acid was dissolved in a HEPES buffer solution (0.01 M, pH = 7.4) to yield a 0.15 M solution. As solubilization of 2,5-dinitrosalicylic acid is slow, it was assisted by sonication. 0.1 M EDC (1-ethyl-3-(3-dimethylamino propyl)carbodiimide and 0.1 M NaNHS (N-hydroxysulfosuccinimide sodium salt)

were dissolved in the antigen solution. The cystamine-modified electrodes 106 (see Fig. 4) were incubated in this solution for two hours to yield an antigen-cystamine monolayer 108 immobilized on electrode 102. This yielded a typical Δf of the crystal of about -100 Hz (Fig. 6).

5

3. Analysis of small molecules in accordance with the Displacement Method

3.1 General outline of the displacement embodiment

The scheme for analysis of a small assayed molecule is illustrated schematically in Fig. 7. The antigen monolayer 120 is contacted with an antibody 122
10 which then binds to the antigen moieties 124 to yield an antigen- antibody monolayer 126. Measurement of resonance frequency at this stage yields a certain basic frequency f_0 .

Challenging the electrode with a sample comprising antigens 128 causes release of some of the antibodies 122 to yield a soluble antigen- antibody
15 complex 130. This reduces the immobilized mass and consequently the frequency is increased as a result of this antibody displacement to a certain frequency f' . The decrease in frequency is a result of and signifies the presence of the assayed molecule 128 in the medium; the extent of the frequency change depends on the concentration of the assayed molecule in a sample.

20

3.2 Assay Apparatus

An assay apparatus generally designated 150 is shown schematically in Fig. 8. The apparatus 150 comprises a sensing member 152 and an analysis cell 154. The sensing member 152 consists of the quartz crystal 156 sandwiched between two gold
25 electrodes 158 and 160 which are connected through connector 162 to a control utility (not shown) for inducing current in electrodes 158,160 and measuring the resonance frequency of crystal 156.

Sensing member 152 is sandwiched between two O-rings 162 and 164 and has one electrode 160 which faces cell 154. Electrode 160 serves also as the sensing
30 surface of sensing member 152.

Cell 154, typically having a volume of 1 ml or less, has entry and exit ports 170 and 172, respectively which allow rinsing of cell 154. Inlet port 170 is connected through duct 174 to a peristaltic pump 176 which pumps fluid from reservoir 178 into cell 154; outlet port 172 is connected to a duct 180 which drains fluid to a drain (not shown).

Cell 154 has also a liquid injection port 184 for injection of samples to be assayed. As will no doubt be appreciated, rather than injection port, the cell may comprise another port which will permit propelling of a liquid into the cell directly from a specimen sampling device (not shown).

3.3 Assaying of samples using the apparatus of Fig. 8

The sensing member is mounted in the cell, the cell volume is filled with a solution, e.g. 0.01 M phosphate buffer, pH 7.4 comprising also 0.1 M NaCl ("*NaCl-containing PBS*"). The sensing member is allowed to equilibrate until a constant frequency of the crystal is observed.

The sensing surface in accordance with this embodiment, where the neutralizing agent is an antibody, comprises an antigen-antibody monolayer. Electrode 160 may be provided, *a priori* with antibodies bound thereon, although alternatively, the antibody may be bound to the sensing surface *in situ*. In the latter case, an antibody solution, e.g. 50 μ l of a 1 mg/ml antibody solution is injected into the cell and permitted to incubate with the electrode for a time to allow binding, e.g. 15 minutes. Association of the antibody to the electrode can be monitored by the decrease in the resonance frequency of the crystal (see Fig. 9 and the accompanying description below). The cell may then be rinsed with several cell volumes of a solution, e.g. a buffer solution, propelled by means of peristaltic pump 176. The electrode is then allowed to calibrate until the resonance frequency remains constant for a period of time.

Samples may then be injected into the cells. Detachment of the antibody from the monolayer is evidenced by a frequency increase of the crystal (see exemplary Fig. 10 and the accompanying description below). After each positive sample the cell is

flushed with a buffer solution and allowed to re-equilibrate to reach a constant frequency.

It was found that usually a single electrode can be used consecutively for several positive samples, e.g. three positive samples. After several positive samples,
5 the antibody monolayer has to be reformed.

In the specific example of the antibodies which will be described below, it was found that three positive 2,4-DNT samples in the concentration range of about 0.3-7 ng/ml can be analyzed with a single electrode prior to the need to recharge the electrode with antibodies. With the same antibody interface, typically three TNT
10 positive samples in the concentration range of 6-65 pgr/ml can be analyzed with a single electrode prior to the need for recharging. A single electrode can typically be recharged several times, e.g. three, until the sensing member has to be changed. It should be noted that in typical use, most samples are expected to be negative and thus the requirement to occasionally recharge the electrode or change the sensing member
15 is not a serious drawback.

3.4 Analysis of 2,4-DNT

DNP-Ab was charged on the antigen monolayer as described above. Fig. 9 shows the crystal frequency decrease upon association of the anti-DNP antibodies
20 (DNP-Ab) to the monolayer. To obtain full charging of the monolayer, the electrode was interacted with the DNP-Ab for 15 minutes. The cell was then rinsed with the buffer solution.

2,4-DNT was dissolved in ethyleneglycol monomethyl ether, 100 μ l and diluted with the NaCl-containing PBS to 1 ml. The solution was diluted repeatedly with the
25 buffer solution to the desired concentration.

Injection of 2,4-DNT sample (50 μ l) to the cell, that yields in the cell a concentration of 2,4-DNT of about 20 ng/ml⁻¹, displaces the DNP-Ab. This can be seen in Fig. 10 which shows the crystal frequency changes following such injection of 2,4-DNT. A frequency increase of about $\Delta f = 16$ Hz was observed.

3.5 Analysis of 2,4-DNT by IgG TTDA 5B3-Ab

An antigen monolayer electrode was treated for 15 mins. with the Ab. Fig. 11 shows the crystal frequency decrease upon saturation of the monolayer. A decrease of about $\Delta f = -100$ Hz was observed. The charged electrode was rinsed for 3 mins with
5 the buffer solution and its resonance frequency was stabilized.

50 ml of stock solution of 2,4-DNT, prepared as described under 3.4 was injected into the cell to yield the desired concentration. The crystal frequency increased by about $\Delta f = -35$ Hz upon injection of 2,4-DNT to a concentration of 6.25 ng/ml into the cell, as can be seen in Fig. 12 (curve a).

10 The electrode was then rinsed for three mins. with the buffer solution and its frequency was re-stabilized. A sample of 2,4-DNT was injected again into the cell to yield the sum concentration of 6.25 ng/ml. A frequency increase of about $\Delta f = -30$ Hz was observed as can be seen in Fig. 12 (curve b). The electrode was rinsed again and its frequency was stabilized. A 2,4-DNT sample was injected into the cell to yield a
15 concentration of 312 pgr/ml. The crystal frequency increases again by about $\Delta f = -15$ Hz, as can be seen in Fig. 12 (curve c).

This example shows that the antigen monolayer electrode charged with the Ab is reusable for at least three positive samples of 2,4-DNT.

20 3.6 Analysis of TNT by IgG TTDA 5B3-Ab interface

3.6.1 Repeated assaying of TNT

An electrode of a sensing member was charged with Ab in an identical manner to that described under 3.5.

A TNT stock solution was prepared similarly to the manner of preparation of
25 the 2,4-DNT stock solution in Example 3.5. A 50 ml sample of the TNT stock solution was injected into the cell to yield a concentration of 6.25 pgr/ml in the cell. This resulted in a time-dependent frequency increase of up to about $\Delta f = 7$ Hz (Fig. 13, curve a). After rinsing the cell and stabilization of the crystal frequency, a second sample of TNT was injected to the cell, yielding a concentration of 62.5 pgr/ml¹.

This resulted in a time-dependent frequency increase of the crystal up to about $\Delta f = 16$ Hz (Fig. 13, curve b).

The analysis of TNT can be repeated for at least three times after charging the monolayer with the Ab.

5 This example demonstrates the possibility of reusing the electrode for at least three samples of TNT in this concentration range.

3.6.2 Recharging of the monolayer with IgG TTDA 5B3-Ab

10 The monolayer electrode employed for the analysis of two consecutive TNT samples (6.25 and 62.5 pgr/ml⁻¹, respectively) was rinsed and recharged with IgG TTDA 5B3 Ab (see Fig. 14) as described in Example 3.6.1. Fig. 15 shows the crystal frequency increase upon treatment of the electrode with a TNT sample, at a final concentration within the cell of 6.25 pgr/ml. A frequency increase up to about $\Delta f = 18$ Hz was observed indicating that the Ab was displaced from the interface as a result of
15 the presence of TNT in the sample.

This example demonstrates the possibility of recharging the electrode with the Ab and regenerating the active interface for TNT/DNT analysis.

3.7 Amplification of 2,4-DNT detection by DNP-Ab

20 The concept for the amplification of DNT/TNT analysis is schematically outlined in Fig. 16. The antigen monolayer-modified crystal **210** is functionalized by a biotinylated DNT/TNT-Ab **212**. Avidin **214** is further linked to the monolayer yielding an Ab-avidin complex monolayer **216** on the crystal. Challenging the functionalized electrode with a DNT/TNT antigen **218** results in the displacement of the Ab-avidin
25 complex **220** from the monolayer as a result of binding to the analyte-antigen. The increased mass of the dissociated material from the monolayer allows the detection of minute quantities of displaced Ab and consequently the sensitivity of the analysis of DNT/TNT is enhanced. (The difference, Δf , between the basic resonance frequency, f_0 and that after dissociation, f' will increase.

As will be appreciated, this example of assaying the explosive TNT or DNT is a mere illustration of the more general concept of assaying small molecules, which may be other explosive molecules as well as non-explosive molecules.

5 3.7.1 Preparation of biotinylated DNP-Ab

0.5 mg of the DNP-Ab was introduced into 1 ml of the NaCl-containing PBS. 5 mg Biotinamidocaproate N-hydroxy-succinimide ester, was added to the mixture. The solution was mixed at room temperature for 1 hour and then dialyzed against the NaCl-containing PBS for 15 hours. Dialysis was performed at 0°C. The
10 resulting 7.5 ml dialyzed antibody solution was concentrated to 2 ml using an Amicon™ (Amicon, U.S.A.) filter.

3.7.2 Charging of the antigen monolayer with the DNP-Ab-avidin complex

15 The antigen monolayer electrode was charged with the 0.1 mg/ml biotinylated DNP-Ab as described in Example 3.4 After charging of the electrode with the Ab, the cell was rinsed with a PBS (0.01 M, pH = 7.4) buffer solution for 3 mins. The electrode frequency was then stabilized and 50 µl of a 0.01 M avidin solution in the PBS buffer were injected into the cell. Fig. 17 shows the crystal frequency changes
20 upon association of the biotinylated Ab to the antigen monolayer. A frequency decrease of about $\Delta f = -50$ Hz was observed.

Fig. 18 shows the time-dependent frequency decrease of the crystal charged with the biotinylated Ab upon interaction with avidin. The crystal frequency decreases by about $\Delta f = -120$ Hz, indicating the formation of the Ab-avidin complex on the
25 monolayer. The resulting charged electrode was rinsed with PBS and the crystal frequency was stabilized.

3.7.3 Analysis of 2,4-DNT by the electrode functionalized by the Ab-avidin complex monolayer

30

A stock solution of 2,4-DNT was prepared as described in Example 3.4. A 50 ml sample of the 2,4-DNT stock solution was injected into the cell to yield a

concentration of 2.7 ng/ml within the cell. Fig. 19 shows the crystal frequency increase upon interaction of the electrode that contains the DNP-Ab-avidin layer with the 2,4-DNT sample. A frequency increase of about $\Delta f = 30$ Hz is observed, indicating the displacement of the Ab-avidin complex from the monolayer.

5 It should be noted that a sensor of the kind used in Example 3.4 did not show any frequency increase upon interaction with 2,4-DNT at a concentration of 2.7 ng/ml. The frequency increase of such a concentration is only observed in the presence of the biotinylated Ab-avidin complex, indicating that the complexed Ab-avidin layer enhances the sensitivity of 2,4-DNT detection (about 10 fold sensitivity enhancement
10 as compared to the DNP-Ab monolayer alone).

3.8 Integrated assay assembly

An assembly 300 in accordance with an embodiment of the invention, which was used in some experiments reported below, can be seen in Fig. 20. The sensing
15 apparatus 302 is provided with a sensing member 304, with a similar design to that of the apparatus shown in Fig. 8, and an assay cell 306. Sensing apparatus 304 is connectable to an oscillator circuit unit 308, these two components shown here, for the sake of illustration as being detached from one another. Apparatus 302 is included within a Faraday cage 310.

20 Cell 306 is provided with a fluid inlet port 312 and a fluid outlet port 314. Inlet port 312 is connected to a feedline 316 which is provided with a pump 320 which is under flow control of controller 322. Pump 320 can feed a solution, e.g. a buffer solution, from reservoir 324 into cell 306. Outlet port 314 is connected through ducting line 330 to fluid drain 332.

25 Cell 306 is further provided with a sample inlet port 334 linked to a three-port injection valve fitted with a 20 μ l sample-injection loop 338 (having a design typical to injection loops used in liquid chromatography) which may be linked either to a dispenser line 340, e.g. one fitted with a syringe driver 342 which is controlled by a controller 344. Alternatively, in another embodiment, line 340 may also be linked
30 directly to reservoir 324. The syringe (not shown) of the syringe driver 342 is typically

filled with a buffer solution. Oscillator circuit unit 308 is connected to a QCM device 350 linked to a computer 352. Computer 352 is also linked to controllers 322 and 344.

The following are examples of experiments performed in such a system.

5 3.8.1 Charging of the electrode in the micro-cell

The electrode modified by the antigen monolayer was placed in the micro-cell and the cell volume (50 μ l) was filled with the NaCl-containing PBS by pumping buffer from the buffer reservoir at a flow rate of 0.5 ml/min. The electrode resonance frequency was allowed to stabilize and the 20 μ l injection loop was filled with the 0.1
10 mg/ml IgG TTDA 5B3 Ab solution. The Ab solution was injected into the cell using the buffer solution in the syringe dispenser to drive out the loop content. 50 μ l of the buffer solution were driven by the syringe dispenser at a rate of 60 μ l. This volume is that required to drive the Ab solution from the loop and the dead volume of the solution in the connecting pipe into the analyzing cell volume. Fig. 21 shows the
15 crystal frequency changes upon charging the electrode with the Ab. After about 10 mins. of interaction, the electrode was fully charged. The Ab-charged electrode and the injection loop were washed for 4 mins. with a solution from the buffer reservoir, at a flow-rate of 0.5 ml/min., and allowed to equilibrate to a constant frequency. The electrode was then ready for the analysis of DNT/TNT samples.

20

3.8.2 Analysis of TNT by the IgG TTDA 5B3 Ab-modified electrode in the micro-cell

A 250 pgr/ml TNT sample was prepared by the dilution procedure described in
25 Example 3.6. The 20 μ l loop in the injector was filled with the sample. The sample was injected into the cell by the syringe driver using 50 μ l of the PBS solution through the loop at a flow-rate of 60 μ l/s. As noted above, this volume is that required to drive the loop content into the cell and to overcome the dead volume of the connecting pipe. The final concentration of TNT within the cell was 100 pgr/ml. Fig. 22 shows the
30 time-dependent frequency changes of the crystal as a result of TNT interaction with

the electrode. After 3 mins. a frequency increase of about 15 Hz was observed. The frequency increase indicated the displacement of the antibody from the monolayer by the TNT.

After completion of the TNT analysis, the injection loop and the cell were washed for 4 mins. with the buffer solution from the reservoir, at flow rate 0.5 ml/min., and the crystal frequency was re-stabilized, and was ready for another assay sequence. It should be noted that the cell and the loop do not require washing if a non-contaminated sample of suspected TNT is injected into the cell.

10 4. Analysis according to the Competition Embodiment

In the description below of this embodiment, specific reference will be made to the case where the neutralizing agent is an antibody and the assayed molecule is DNT or TNT. As will be not doubt appreciated this is an example meant to illustrate the invention and should not be construed as limiting.

15 4.1 Basic Scheme

The general scheme of the competition method is displayed in Fig. 23. The quartz crystal is modified with the DNT/TNT antigen monolayer to yield sensing member 400. A stock solution that contains the antibody 402 at a fixed concentration is used as the probe solution. The analyte sample 404 is mixed with the probe solution. In the absence of the DNT/TNT analyte, the antibody remains free, and interaction of the probe solution with the modified crystal results in the association of the free antibody to the antigen monolayer to yield conjugate 406. This results in a frequency decrease of the crystal. Interaction of a sample that includes the DNT/TNT antigen 404 with the antibody probe solution yields the association 408 of the antigen to the antibody. Subsequent interaction of the probe solution with the crystal does not yield the association of the antibody to the monolayer interface, as the antibody is occupied. No frequency change of the crystal will be observed. Thus, a DNT/TNT-free sample induces a frequency decrease of the crystal where a DNT/TNT positive sample does not affect the crystal frequency. It should be noted that preferably, the molar

concentration of the antibody in the probe solution must be about 15% lower than the desired molar concentration limit (sensitivity) of the analysis.

4.2 Amplification of the analysis by the competition embodiment

5 Fig. 24 outlines the principle of the analysis of DNT/TNT through amplification of response obtained in the competition embodiment. The quartz crystal is modified by the DNT/TNT antigen monolayer to yield sensing member 420 (essentially identical to member 400 in Fig. 23). A solution consisting of a fixed concentration of the antibody 422 and a fixed concentration of the anti-antibody 424
10 which bind to yield conjugate 426 is used as a probe solution. The analyte sample is mixed with the probe solution and incubated for a sufficient time for binding, and then interacted with the antigen-modified crystal. If the analyte sample does not include DNT/TNT, the Ab-anti-Ab complex of the probe solution will associate to the crystal to form immobilized complex 428 resulting in a frequency decrease. Mixing of a
15 positive DNT/TNT sample with the probe solution will generate the antigen-Ab-anti-Ab complex 430 in the probe solution. Interaction of this probe solution with the antigen-modified crystal will not affect the crystal frequency since the sensing Ab is occupied by the antigen. It should be noted that the association of the Ab-anti-Ab complex to the antigen monolayer results in a substantially higher
20 frequency change as compared to that induced by the Ab alone (Example 4.1) since the mass of the associated complex is higher. This allows observation of the higher values of frequency changes and decrease of the concentration of the analyzing Ab in the probe solution. Decrease of the Ab concentration in the probe solution permits to detect lower antigen concentration and to enhance the sensitivity of sensing.

25

4.3 Experimental Results

4.3.1 Assembly of the antigen monolayer on the quartz crystals

The 3,5-dinitrosalicylic acid was covalently linked to a cystamine monolayer assembled on the Au-electrodes associated with the quartz crystal as
30 described in Example 2.

4.3.2 Antibodies used in the competition and amplified competition methods

Mouse IgE anti-dinitrophenyl (DNP-Ab) was used as the probe antibody. Goat
5 anti-mouse Fc antibodies were used as the amplifying anti-Ab.

4.3.3 Analysis of 2,4-Dinitrophenol (DNP) by the competition method

The configuration of the embodiment is seen in Fig. 24.

10 A stock solution of 0.3 ng/ml dinitrophenol, in NaCl-containing PBS was prepared. From the stock solution, 10 μ l were withdrawn into a vial and 10 μ l DNP-Ab solution from 11×10^{-10} M stock solution were added to the vial. The mixture was incubated for 15 mins. The total amount of dinitrophenol in the vial was 3.1×10^{-12} g. The resulting solution was injected into 1 ml of 0.01 M aqueous
15 phosphate buffer solution, that included 0.1 M NaCl, pH = 7.4, were incubated with 10 μ l of the 11×10^{-10} M DNP-Ab solution. The resulting mixture was injected to the cell solution as described above and the frequency changes of the crystal over time were followed. This reference system does not include the dinitrophenol analyte.

Fig. 25 (curve a), shows the frequency changes of the crystal in the reference
20 system lacking dinitrophenol. A frequency decrease of about $\Delta f = -30$ Hz indicates the absence of the analyte in the sample. With the system that includes 3.1 pgr/ml dinitrophenol, a frequency decrease of only (-5.0)-(-8.0) Hz is observed (results not shown) indicating that the probe antibody is occupied.

25 4.3.4 Analysis of 2,4-dinitrophenol (DNP) by the amplified competition method

A solution of 6.7×10^{-6} M DNP-Ab and 6.7×10^{-6} M anti-Ab was mixed and incubated for 15 mins. The solution was repeatedly diluted to yield a stock solution of 11×10^{-10} M of the DNP-Ab/anti- Ab complex that was used as the probe Ab/anti-Ab
30 solution.

10 μ l of a DNP stock solution in an NaCl-containing PBS was incubated with 10 μ l of the DNP-Ab and anti-Ab stock solution for 15 mins. The resulting mixture was injected into the cell as described in Example 4.3.3.

5 In a reference system, 10 μ l of the phosphate buffer was mixed with the 10 μ l of the DNP-Ab/anti-Ab stock solution and the resulting mixture was injected into the cell as described in Example 4.3.3.

Fig. 25 (curve b), shows the frequency changes of the crystal upon injection of the DNP-Ab/anti-Ab probe solution lacking the DNP antigen. A frequency change of $\Delta f = -85$ Hz is observed, indicating the association of the vacant DNP-Ab/anti-Ab to the crystal interface. Fig. 25 (curve c), shows the frequency changes or the crystal upon injection of the DNP-Ab/anti-Ab probe solution that includes 3.1×10^{-12} g of DNP. Only a slight frequency change of about $\Delta f = -12$ Hz, is observed, implying that the DNP-Ab/anti-Ab complex is occupied by the DNP analyte.

15 4.3.5 Analysis of 2,4-DNT by the amplified competition method using DNP-Ab and Anti-Ab as probes

10 mg of 2,4-DNT were dissolved in 1 ml of ethylene glycolmonomethyl ether. 100 μ l of this solution were diluted to 1 ml with a 0.01 M phosphate buffer solution, pH = 7.4, that included 0.1 M NaCl. This solution was repeatedly diluted to achieve the desired concentration of the analyte sample.

A 100 ml of 54×10^{-10} M solution of DNP-Ab and anti-Ab were mixed and incubated for 15 mins. to yield the DNP-Ab/anti-Ab probe solution.

10 μ l of the 2,4-DNT stock solution that included 4.39×10^{-12} g of 2,4-DNT were mixed with 10 μ l of the DNP-ab/anti-Ab probe solution and incubated for 15 mins. The resulting mixture was injected into the measuring cell that included 1 ml of the phosphate buffer. The frequency change of the crystal as a function of time is seen in Fig. 26, (curve a), showing a final minute frequency decrease of about $\Delta f = -2$ Hz. Fig. 26 (curve b), shows the results of a similar experiment performed for a second time with the same electrode.

As reference system, 10 μl of the phosphate buffer was treated with 10 μl of the DNP-Ab/anti-Ab probe solution as described above. The mixture was then injected into the measuring cell as described for the previous examples. This system lacks the analyte antigen (2,4-DNT) and hence the DNP-Ab/anti-Ab complex stays vacant and is capable of binding to the antigen monolayer of the crystal. Fig. 26 (curve c), shows the crystal frequency changes with time upon injection of this reference solution. The reference solution was injected to the same electrode that was previously employed to twice detect the 2,4-DNT analyte samples. A frequency decrease corresponding to $\Delta f = -18$ Hz was observed, implying that the vacant DNP-Ab/anti-Ab complex associates to the antigen monolayer assembled onto the crystal.

Some conclusions may be drawn from this set of experiments as follows:

(a) A 2,4-DNT-containing sample does not significantly affect the frequency of the crystal. A non-contaminated analyte sample stimulates a frequency change of $\Delta f = -18$ Hz. Any sample showing a frequency change of less than $\Delta f = -5$ Hz may be regarded as a 2,4-DNT contaminated sample.

(b) The method permits successive use of the electrode for 2,4-DNT positive samples. A negative 2,4-DNT sample ruins the electrode since the sensing interface is saturated. After each negative sample, the sensing electrode must be exchanged. This embodiment is particularly useful in the case where a large proportion of the samples are expected to be contaminated with an explosive.

4.3.6 Analysis of DNT-isomers by the amplified competition method using the Dinitrosalicylic acid antigen monolayer and DNP-Ab/anti-Ab as probe

A similar antigen-monolayer-modified crystal was examined as a potential sensing interface for other DNT isomers. The set of experiments described in Example 4.3.5 was performed for 2,4-DNT (78 pgr/ml^{-1}), 1,4-DNT (94 pgr.ml^{-1}), 2,6-DNT (78 pgr/ml^{-1}) and 3,4-DNT (75 pgr/ml^{-1}),

Figs. 27A to 27C shows the frequency changes of the crystals in the presence of different DNT-isomer (2,4-DNT, 1,4-DNT and 2,6-DNT, respectively) positive samples (curves a in all these figs.). The frequency changes of the crystals upon their interaction with positive samples of the different isomers, and with the respective reference systems that include the vacant DNP-Ab/anti-Ab probe solution is shown in the following Table 2:

Table 2

Analyte	Concentration pgr/ml	ΔF with analyte	ΔF without analyte
2,4-DNT	78	-6	-16
1,4-DNT	94	07	-18
2,6-DNT	78	03	-12

The results reveal that the isomers 2,4-DNT, 1,4-DNT and 2,6-DNT are recognized by the DNP-Ab/anti-Ab probe solution.

A similar experiment with 3,4-DNT indicated that this isomer is not recognized by the DNP-Ab/anti-Ab probe solution. Thus for this isomer a different antibody will be required.

4.3.7 Stability studies of the manufactured antigen-monolayer- modified crystal

A series of 10 crystals that include Au-electrodes were parallel treated in a manner as described in Example 2. The electrodes were dried with an argon and stored under argon at 4°C.

At different time intervals different electrodes were examined for their sensing activity of TNT as analyte and the displacement embodiment as the sensing procedure. The respective electrode was charged with the IgG TTDA 5B3 antibody and 100 pgr/ml of TNT were injected to the cell as detailed in Example 3.

The frequency of the crystal was followed as a function of time to characterize the displacement of the antibody from the sensing interface.

Figs. 28A-28C show the frequency changes of different electrodes which had been stored for , respectively, 7 days, 15 days and 28 days, with TNT (100pgr/ml). All of the electrodes reveal comparable activities indicating that the sensing interfaces on the crystals are stable in storage. Electrodes stored for at least 75 days under the same conditions retained their sensing activities.

5. Analysis of DNT/TNT by the basic filtration embodiment

This embodiment is outlined generally in Fig. 29.

The quartz crystal is modified by biotin, and avidin is adsorbed to the base monolayer to generate the biotin-avidin sensing interface 500. A filtering support 502 is prepared in the form of an inert solid, coating or filtering material to which the antigen is linked, e.g. by covalent bonding.

The probing antibody is modified by biotin to yield a biotin- functionalized antibody 504. The biotinylated antibody (Ab-B) 506 is solubilized at a fixed concentration to generate the probe solution.

For analysis of DNT/TNT in samples, the ample is first reacted with the antibody probe solution for a time sufficient to ensure binding between the antibodies and the explosive if present in the sample. The probe solution is then passed through or interacted with the filtering support and subsequently introduced into the measuring cell and interacted with the quartz crystal sensing member. As illustrated on the left side of Fig. 29, a clean analyte sample, lacking DNT/TNT, will result in the association of the probe antibody 504 to the filtering support 502. The resulting solution introduced to the cell will lack the Ab-B 504 and the crystal frequency will not change. In the case of a positive DNT/TNT, illustrated on the right side of Fig. 29, the antibody will bind the DNT/TNT antigen. Interaction of the probe solution with the crystal results in the association of the Ab-B to the biotin-avidin monolayer and gives rise to frequency decrease of the crystal.

It is to be noted that the characteristic high sensitivity of the competition embodiment is also a feature of the basic filtration embodiment. Additionally the sensing surface of the crystal is deteriorated only by positive DNT/TNT sample

similarly as in the case of the competition embodiment. Furthermore a positive DNT/TNT sample is reflected by a frequency decrease in contrast to the competition embodiment where a positive test does not influence the crystal frequency. This reduces the probability of false positive results of the competition embodiment.

5 The following are some specific examples of this embodiment.

5.1 Modification of the quartz crystal with a biotin/avidin monolayer

Fig. 30 outlines the stepwise modification of the Au-surfaces associated with the quartz crystal to yield the biotin-avidin layer. The Au-surfaces were cleaned and
10 modified by the primary cystamine monolayer as described in Example 2. The cystamine-modified electrode was interacted with an aqueous solution of 0.01 M biotinamidocaproate N-hydroxy- succinimide ester for 1 hour. The resulting biotinylated monolayer-modified crystal was washed, dried and introduced into the measuring cell that included 0.01 M phosphate buffer, pH = 7.4. An avidin solution
15 was injected into the cell to yield a concentration of 6.5×10^{-3} M in the cell to yield a biotin-avidin monolayer. Fig. 31 shows the resonance frequency change of the crystal with time as a result of the charging of the biotin monolayer with avidin. The cell volume was then washed with the phosphate buffer solution. The system is now charged for analysis of the DNT/TNT analytes.

20

5.2 Preparation of filtering supports

Two different types of filtering supports were prepared:

(a) Glass capillaries, 8 cm in length, 1.5 mm in diameter, were filled with 0.5 M solution of 3-aminopropyltriethoxysilane in toluene and introduced into the
25 same solution. The solution was heated to 85°C for 15 hours. The resulting capillaries were washed with toluene and then with ethanol and water. The capillaries were then filled with an aqueous phosphate buffer solution, pH = 7.4, that included 0.01 M 3,5-dinitrosalicylic acid, 0.1 M 1-ethyl-3-(3-dimethylamine propyl)carbodiimide (EDC), and 0.1 M N-hydroxysulfosuccinimide sodium salt. The capillaries were

allowed to react with this solution for 2 hours. The resulting capillaries were washed with a 0.01 M phosphate buffer solution, pH = 7.4, and dried.

(b) The base of a vial, 0.5 cm diameter, 1.0 cm height, were coated with a gold layer by vacuum deposition. The gold surface was modified by the 3,5-dinitrosalicylic acid antigen monolayer by a procedure similar to that described in Example 2 for the assembly of the antigen monolayer on the Au-surfaces associated with the quartz crystal.

5.3 Preparation of biotinylated DNP-Ab

The DNP-Ab was modified with biotin as described in Example 3.7.1.

5.4 Analysis of 2,4-DNT by the competition method and filtering support cartridge

10 μ l of a diluted 2,4-DNT solution, that included 0.156 ng of 2,4 DNT were added to 10 μ l of a 4.2×10^{-8} M biotinylated DNP-Ab solution (DNP-Ab-B). The mixture was incubated for 10 mins. and then introduced into the modified glass capillary. The solution was incubated in the glass capillary for an additional 10 mins. The solution from the capillary was then injected into the measuring cell, and the time-dependent frequency changes of the crystal was recorded.

20 As a reference system that lacks the DNT-analyte, 10 μ l of the 4.2×10^{-8} M DNP-Ab-B probe solution, and the resulting mixture was treated in the capillary filtering column and injected into the cell in the same manner as above.

Fig. 32 (curve a), shows the crystal frequency change upon injection of the reference system. No significant frequency changes was observed ($\Delta f < -5.0$ Hz), implying that no DNP-Ab-B associated to the biotin/avidin monolayer-modified crystal. The results indicate that the DNP-Ab-B is filtered by the antigen-modified capillary. Fig. 32 (curve b), shows the frequency changes of the same crystal upon injection of the 2,4-DNT analyte sample. A frequency decrease of $\Delta f = -35$ Hz is observed, indicating that the antigen-occupied DNP-Ab passes through the filtering column and associates to the biotin-avidin monolayer.

6. Analysis of DNT/TNT by the enzyme embodiment

This embodiment is essentially a modification of the basic filtration embodiment described in Example 5 and is outlined in Figs. 33 and 34.

The electrode on the quartz crystal **600** is modified by anti-horseradish peroxidase antibody (anti-HRP) **602** to generate the sensing surface for HRP-Ab conjugate **604**. A filtering support **608** may be a solid matrix, a filtering material, to which antigen **610** is linked.

The probing antibody is modified by HRP to yield a HRP-Ab conjugate **612**. The HRP-Ab conjugate **612** is solubilized in an aqueous solution yielding the probe solution.

For analysis, a sample is first interacted with the HRP-Ab probe solution and incubated for a time sufficient to allow binding of the antibody to explosive molecules in the sample (if any). The probe solution is then passed through or interacted with the filtering support **608** and subsequently introduced into the measuring cell and allowed to interact with the modified quartz crystal. The case of a clean sample is illustrated on the right-hand side of Fig. 33, while the case of a DNT/TNT-contaminated sample is illustrated on the left-hand side of Fig. 33. A clean sample lacking DNT or TNT, will result in the association of the probe HRP-Ab to the filtering support **608** (due to the bioaffinity interaction between the antigen associated with the filtering support (an antibody that is part of the HRP-Ab conjugate). The resulting solution introduced to the cell will lack the HRP-Ab and the crystal's mass will thus not change. Consequently, there will be no resonance frequency change (see illustrative graph **620**).

A positive DNT/TNT sample will result in binding of the antigen to the antibody to yield conjugate **614** in the probe solution. The filtering support will not bind the HRP-Ab since it is associated with the DNT/TNT antigen. Interacting the probe solution with the crystal will then result in association of the HRP-Ab conjugate **614** to the anti-HRP monolayer (due to the bioaffinity interaction between anti-HRP on the surface and HRP that is part of the HRP-Ab conjugate). This results in a frequency decrease of the crystal (see illustrative graph **622**).

When the crystal carrying the anti-HRP/HRP-Ab/TNT complex **630** is incubated in a solution of hydrogen peroxide and 4-chloronaphthol, the HRP catalyzes a reaction **632** in which this substrate (4-chloronaphthol) is oxidized by the hydrogen peroxide giving rise to an insoluble product which forms a precipitate **634** on the electrode's surface. The formation of the insoluble product decreases dramatically the frequency of the crystal (see illustrative graph **640** in Fig. 34) that results in a significant amplification of the primary signal.

The specific biocatalytic reaction, in addition to amplifying the primary signal, serves also as a confirmation to verify the primary signal. In the case of non-specific adsorption of some impurities, the secondary amplified signal will not arise. Thus, the secondary decrease in the crystal's frequency that is biocatalyzed by the enzyme (in a specific example – HRP) can serve as the confirmation of the bioaffinity coupling of the HRP-Ab conjugate to the crystal's surface. A wrong primary frequency change resulting from absorption of non-catalytic active species can thus be easily discriminated. The amount of the precipitate, and hence the extent of the signal amplification, may be controlled by either the surface concentration of the HRP and by the time of the crystal incubation in the developing solution which contains hydrogen peroxide and 4-chloronaphthol. It is clear that the extent of amplification can thus be easily controlled by any one of the incubation times, e.g. the time in which the HRP is permitted to catalyze the developing reaction.

Many different enzymes and respective substrates which produce insoluble products may be used in this embodiment. The following are some examples:

- i. Horseradish peroxidase (HRP) or microperoxidase-11, with several substrates including 4-chloronaphthol, 3,3'-diaminobenzidine tetrahydro- chloride, or 3-amino-9-ethyl carbazol.
- ii. Alkaline phosphatase with the substrates: 5-bromo-4-chloro-3'-indolyphosphate p-toluidine or nitro-blue tetrazolium chloride.
- iii. Glucose oxidase with the substrates: nitro-blue tetrazolium chloride, tetranitroblue tetrazolium.

- iv. Galactosidase with the substrate: 5-bromo-4-chloro-D-galactopyranoside.

Obviously, as will be appreciated, in each case the surface of the electrode should carry the respective anti-enzyme antibody.

CLAIMS:

1. Apparatus for detecting small assayed molecules in a sample, comprising:
 - (a) a sensing member comprising a piezoelectric crystal having at least one
5 sensing surface which can interact with a medium in contact therewith a by
either binding a first indicator agent from the medium, or by releasing a
second indicator agent originally immobilized on the sensing surface into the
medium; the medium being either the sample in which case the assayed
molecule present, causes the release of the second indicator agent from the at
10 least one sensing surface, or being a treated sample preparation obtained by
reacting the sample with one or both of a reagent solution or
sample-processing hardware, such that said medium comprises a first
indicator agent or a second indicator agent-releasing species at a
concentration of said agent or species which is in correlation to the
15 concentration of the assayed molecule in the sample, the binding or release
resulting in a change of mass of the sensing surface;
 - (b) a testing cell for holding said medium and bringing it into contact with said at
least one sensing surface; and
 - (c) an electric or electronic utility for inducing vibrations in the piezoelectric
20 crystal and measuring resonance frequency thereof, a change in resonance
frequency after contact between the sensing surface and the medium,
indicating presence of the explosive in the sample.
2. An Apparatus according to Claim 1, wherein said assayed molecule is an
explosive molecule.
- 25 3. An apparatus according to Claim 1 or 2, wherein the degree of change in
resonance frequency serves as a measure of the level of said assayed molecule in
said sample.
4. An apparatus according to Claim 1 or 2, wherein said at least one sensing
surface carries capturing agents which bind to neutralizing agents at an assayed
30 molecule-binding domain of the neutralizing agent.

5. An apparatus according to Claim 4, wherein said capturing agents are assayed molecules residues or moieties and said neutralizing agents comprise first antibodies which bind to the assayed molecules.
6. An apparatus according to Claim 2 or 3, wherein said at least one sensing
5 surface carries residues or moieties of the assayed molecules bound to first antibodies which can competitively bind to soluble assayed molecules in a medium in contact with the at least one sensing surface, whereby in the presence of the assayed molecules in said medium the antibodies are released from the at least one sensing surface.
- 10 7. An apparatus according to Claim 5 or 6, wherein the first antibodies are bound or complexed to a mass-increasing agent.
8. An apparatus according to Claim 7, wherein said mass increasing agent comprises a second antibody which binds to said first antibody or comprise avidin or streptavidin bound to a biotin residue conjugated to the first antibody.
- 15 9. An apparatus according to any one of Claims 5-8, wherein said first antibodies are monoclonal antibodies.
10. An apparatus according to Claim 7, wherein said monoclonal antibody has the binding characteristics of the 5B3 monoclonal antibody.
11. An apparatus according to Claim 8, wherein said antibody is said 5B3
20 monoclonal antibody.
12. An apparatus according to any one of Claims 1-11, wherein said assayed molecule is DNT or TNT.
13. A system for detecting small assayed molecules in a sample comprising an apparatus according to any one of Claims 1-12 and one or both of reagents and
25 hardware for processing said sample or for introducing it into said cell.
14. A system according to Claim 13, wherein said hardware comprises a flow system for propelling a medium comprising the sample into said cell.
15. A system according to Claim 13 or 14, comprising:

- (a) a sensing member comprising a piezoelectric crystal having at least one sensing surface which carries residues or moieties of said assayed molecules bound to anti-assayed molecule antibodies;
- (b) a testing cell for holding a medium and bringing it into contact with said at least one sensing surface, whereby in the presence of the assayed molecules in the medium, at least some of said antibodies are released into the medium;
- (c) hardware for introducing the sample into the testing vessel; and
- (d) an electric or electronic utility for inducing vibrations in the piezoelectric crystal and measuring resonance frequency thereof, a reduction in resonance frequency after contact between the sensing surface and the medium, indicating presence of said assayed molecule in the sample.
16. A system according to Claim 15, wherein said assayed molecule is an explosive molecule.
17. A system according to Claim 15 or 16, wherein the anti-assayed molecule antibody is a monoclonal antibody.
18. A system according to Claim 17, wherein the assayed molecule is DNT or TNT and the antibody is that designated herein as 5B3.
19. A system according to any one of Claims 15-18, wherein the anti-explosive antibodies are bound to mass-increasing agents.
20. A system according to Claim 19, wherein said mass increasing agent comprises a second antibody, or an avidin or a streptavidin which bind to a biotin moiety conjugated to the anti-explosive antibody.
21. A system according to Claim 13 or 14, comprising:
- (a) a sensing member comprising a piezoelectric crystal having at least one sensing surface which carries residues or moieties of the assayed molecules;
- (b) a reagent system comprising anti-assayed molecule antibodies;
- (c) a testing cell for holding a medium and bringing it into contact with said at least one sensing surface, whereby in the presence of the assayed molecules in the medium, at least some of said antibodies are released into the medium;

- (d) an arrangement for contacting the sample with said reagent system to obtain a treated sample preparation and for introducing the treated sample preparation into the testing vessel; and
- (e) an electric or electronic utility for inducing vibrations in the piezoelectric crystal and measuring resonance frequency thereof, a decrease in resonance frequency after contact between the sensing surface and the treated sample preparation, indicating that the sample is free of the assayed molecules.
22. A system according to Claim 20, wherein the assayed molecule is an explosive molecule.
- 10 23. A system according to Claim 22, wherein the anti-assayed molecule antibody is a monoclonal antibody.
24. A system according to Claim 22, wherein the assayed molecule is DNA or TNT and the antibody is that designated herein as 5B3.
- 15 25. A system according to any one of Claims 21-24, comprising a mass-increasing agent for binding to said anti-explosive antibodies.
26. A system according to Claim 24, wherein said mass increasing agent comprises a second antibody, or an avidin or a streptavidin which bind to a biotin moiety conjugated to the anti-explosive antibody.
27. A system according to Claim 13 or 14, comprising:
- 20 (a) a sensing member comprising a piezoelectric crystal having at least one sensing surface which carries capturing agents for binding to neutralizing agents;
- (b) a testing cell for holding a medium and bringing it into contact with said at least one sensing surface;
- (c) a reagent system comprising the neutralizing agents which can bind to the
- 25 assayed molecules;
- (d) an arrangement for contacting the sample with the reagent system under condition and for a time permitting binding of the neutralizing agent to the assayed molecules, to obtain a treated sample preparation;

- (e) a filtration system for filtering out from said treated sample preparation neutralizing agents unbound to an explosive molecule to obtain a filtrate essentially devoid of such unbound neutralizing agents;
- (f) arrangement for transfer of said filtrate to said testing cell; and
- 5 (g) an electric or electronic utility for inducing vibrations in the piezoelectric crystal and measuring resonance frequency thereof, an increase in resonance frequency after contact between the sensing surface and the filtrate, indicating presence of the assayed molecules in the sample.
- 10 28. A system according to Claim 27, wherein the assayed molecule is an explosion molecule.
29. A system according to Claim 27 or 28, wherein said neutralizing agent is an anti-explosive antibody.
30. A system according to Claim 29, wherein said antibody is a monoclonal antibody.
- 15 31. A system according to Claim 30, wherein the assayed molecule is DNT or TNT and the monoclonal antibody is that designated herein as 5B3.
32. A system according to any one of Claims 27-31, wherein said filtration system comprises immobilized residues or moieties of molecules of the assayed molecule.
- 20 33. A system according to any one of Claims 27-32, wherein said neutralizing agent is conjugated to a moiety which binds to said capturing agents.
34. A system according to Claim 33, wherein said moiety is a biotin residue and said capturing agent is avidin or streptavidin.
35. A system according to Claim 13 or 14, comprising:
- 25 (a) a sensing member comprising a piezoelectric crystal having at least one sensing surface which carries capturing agents for binding to neutralizing agents;
- (b) a testing cell for holding a medium and bringing it into contact with said at least one sensing surface;

- (c) a reagent system comprising neutralizing agents which can bind to the assayed molecules, said neutralizing agent being conjugated to an enzyme which can catalyze a reaction yielding an insoluble reaction product;
 - (d) an arrangement for contacting the sample with the reagent system under condition and for a time permitting binding of the neutralizing agent to the assayed molecules, to obtain a treated sample preparation;
 - (e) a filtration system for filtering out from said treated sample preparation neutralizing agents unbound to an assayed molecule to obtain a filtrate essentially devoid of such unbound neutralizing agents;
 - (f) arrangement for transfer of said filtrate to said testing cell;
 - (g) an ensemble of reagents and conditions for inducing said enzyme to catalyze the reaction yielding the insoluble reaction product; and
 - (h) an electric or electronic utility for inducing vibrations in the piezoelectric crystal and measuring resonance frequency thereof, an increase in resonance frequency after contact between the sensing surface and the filtrate or after permitting the enzyme to catalyze said reaction, indicating presence of the assayed molecule in the sample.
36. A system according to Claim 35, wherein the assayed molecule is an explosive molecule.
37. A system according to Claim 35 or 36, wherein said neutralizing agent is an anti-explosive antibody.
38. A system according to Claim 37, wherein said antibody is a monoclonal antibody.
39. A system according to Claim 38, wherein the assayed molecule is DNT or TNT and the monoclonal antibody is that designated herein as 5B3.
40. A system according to any one of Claims 36-39, wherein said filtration system comprises immobilized residues or moieties of the assayed molecules.
41. A system according to any one of Claims 36-40, wherein said capturing agent is an immobilized antibody which binds a moiety of said enzyme, such binding not interfering with the catalytic activity of said enzyme.

42 A system according to any one of Claims 35-41, wherein said enzyme is selected from the group consisting of horseradish peroxidase, microperoxidase, alkaline phosphatase, glucose oxidase and galactosidase.

43. A method for detecting a small assayed molecule in a sample, comprising:

- 5 (a) providing a sensing member comprising a piezoelectric crystal having at least one sensing surface which can interact with a medium in contact therewith by either binding a first indicator agent from the medium, or by releasing a second indicator agent originally immobilized on the sensing surface into the medium;
- 10 (b) contacting the at least one sensing surface with a medium being either the sample in which case the assayed molecule if present causes the release of the second indicator agent from the sensing surface, or being a treated sample preparation obtained by reacting the sample with one or both of a reagent solution or sample-processing hardware, such that said medium comprises a
- 15 first indicator agent or a second indicator agent-releasing species, at a concentration of said agent or species which is in correlation to the concentration of the assayed molecule in the sample, the binding or release resulting in a change of mass of the sensing surface;
- (c) inducing vibrations in the piezoelectric crystal and measuring resonance
- 20 frequency thereof; and
- (d) determining whether a change in resonance frequency after contact between the sensing surface and the medium occurred, such change indicating presence of the assayed molecule in the sample.

25 44. A method according to Claim 32, wherein said small molecule is an explosive.

45. A method according to Claim 44, wherein said explosive is DNT or TNT.

46. A method according to any one of Claims 43-45, wherein the degree of change in resonance frequency serves as a measure of the level of said molecule in said sample.

30 47. A method according to Claim 43, comprising:

- (a) providing a sensing member comprising a piezoelectric crystal having at least one sensing surface which carries residues or moieties of the assayed molecules bound to anti-assayed molecule antibodies;
- (b) contacting the sample with the at least one sensing surface;
- 5 (c) inducing vibrations in the piezoelectric crystal and measuring resonance frequency thereof; and
- (d) determining whether there occurred an increase in resonance frequency after contact between the sensing surface and the medium, such increase indicating presence of the assayed molecule in the sample.

10 **48.** A method according to Claim 47, wherein the assayed molecules are explosive molecules.

49. A method according to Claim 47 or 48, wherein the anti-explosive antibody is a monoclonal antibody.

50. A method according to Claim 49, wherein the assayed molecule is DNT or
15 TNT and the antibody is that designated herein as 5B3.

51. A method according to any one of Claims 47-50, comprising binding a mass increasing agent to the antibodies.

52. A method according to Claim 51, wherein said mass increasing agent comprises a second antibody, or an avidin or a streptavidin which bind to a biotin
20 moiety conjugated to the anti-assayed molecule antibody.

53. A method according to Claim 43, comprising:

- (a) providing a sensing member comprising a piezoelectric crystal having at least one sensing surface which carries residues or moieties of the assayed molecules;
- 25 (b) contacting the sample with anti-assayed molecule antibodies and incubating for a time allowing the antibodies to bind to the assayed molecules if present in the sample, to yield a treated sample preparation;
- (c) contacting the treated sample preparation with the at least one sensing surface;

- (d) inducing vibrations in the piezoelectric crystal and measuring resonance frequency thereof; and
- (e) determining whether there occurred a decrease in resonance frequency after contact between the sensing surface and the treated sample preparation, such decrease indicating presence of the assayed molecule in the sample.

54. A method according to Claim 53, wherein the assayed molecule is an explosive molecule.

55. A method according to Claim 53 or 54, wherein the anti-assayed molecule antibody is a monoclonal antibody.

10 56. A method according to Claim 55, wherein the assayed molecule is DNT or TNT and the antibody is that designated herein as 5B3.

57. A method according to any one of Claims 53-56, comprising binding a mass-increasing antibody to the anti-explosive antibody.

15 58. A method according to Claim 57, wherein said mass increasing agent comprises a second antibody, or an avidin or a streptavidin which bind to a biotin moiety conjugated to the anti-explosive antibody.

59. A method according to Claim 43, comprising:

- (a) providing a sensing member comprising a piezoelectric crystal having at least one sensing surface which carries capturing agents for binding to neutralizing agents;
 - (b) contacting the sample with neutralizing agents which can bind to the assayed molecules under condition and for a time permitting binding of the neutralizing agents to the assayed molecules, to obtain a treated sample preparation;
 - 25 (c) filtering the treated sample preparation through filtration system to filter out neutralizing agents unbound to the assayed molecules to obtain a filtrate essentially devoid of such unbound neutralizing agents;
 - (d) contacting said filtrate with the at least one sensing surface;
 - (e) inducing vibrations in the piezoelectric crystal and measuring resonance frequency thereof; and
- 30

(f) determining whether there occurred a increase in resonance frequency after contact between the sensing surface and the treated sample preparation, such increase indicating presence of the assayed molecules in the sample.

5 60. A method according to Claim 59, wherein the assayed molecules are explosive molecules.

61. A method according to Claim 59 or 60, wherein said neutralizing agent is an anti-explosive antibody.

62. A method according to Claim 61, wherein said antibody is a monoclonal antibody.

10 63. A method according to Claim 62, wherein the assayed molecule is DNT or TNT and the monoclonal antibody is that designated herein as 5B3.

64. A method according to any one of Claims 59-63, wherein said filtration system comprises immobilized residues or moieties of molecules of the explosive.

15 65. A method according to any one of Claims 59-64, wherein said neutralizing agent is conjugated to a moiety which binds to said capturing agents.

66. A method according to Claim 65, wherein said moiety is a biotin residue and said capturing agent is avidin or streptavidin.

67. A method according to Claim 43, comprising:

20 (a) providing sensing member comprising a piezoelectric crystal having at least one sensing surface which carries capturing agents for binding to neutralizing agents;

(b) contacting the sample with neutralizing agents which can bind to the assayed molecules, said neutralizing agent being conjugated to an enzyme which can catalyze a reaction yielding an insoluble reaction product, for a time
25 permitting binding of the neutralizing agent to the assayed molecules, to obtain a treated sample preparation;

(c) filtering the treated sample preparation through filtration system to filter out neutralizing agents unbound to the assayed molecules to obtain a filtrate essentially devoid of such unbound neutralizing agents;

30 (d) contacting said filtrate with the at least one sensing surface;

- (e) applying condition permitting the enzyme to catalyze the production of the insoluble reaction product;
- (f) inducing vibrations in the piezoelectric crystal and measuring resonance frequency thereof; and
- 5 (g) determining whether there occurred a decrease in resonance frequency after contact between the sensing surface and said filtrate or after application of said conditions, such decrease indicating presence of the assayed molecules in the sample.

10 68. A method according to Claim 67, wherein the assayed molecule is an explosive molecule.

69. A method according to Claim 67 or 68, wherein said antibody is a monoclonal antibody.

70. A method according to Claim 69, wherein the assayed molecule is DNT or TNT and the monoclonal antibody is that designated herein as 5B3

15 71. A method according to any one of Claims 68-70, wherein said filtration system comprises immobilized residues or moieties of said assayed molecule.

72. A method according to any one of Claims 68-71, wherein said capturing agent is an immobilized antibody which binds a moiety of said enzyme, such binding not interfering with the catalytic activity of said enzyme.

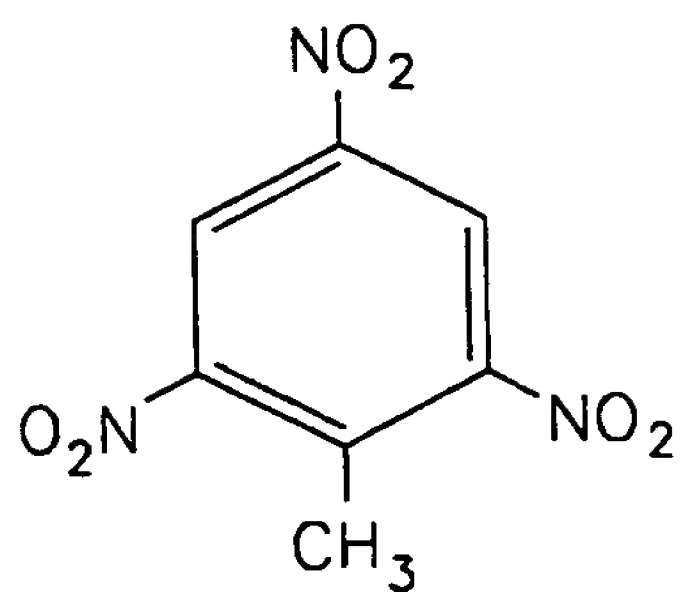
20 73. A method according to any one of Claims 68-72, wherein said enzyme is selected from the group consisting of horseradish peroxidase, microperoxidase, alkaline phosphatase, glucoseoxidase and galactosidase.

74. A protein comprising an antigen-binding portion having two cooperating peptide sequences of Fig. 3A and Fig. 3B or an altered portion where at least one of
25 the peptide sequences is an altered sequence, being a sequence of Fig. 3A or 3B in which one or more amino acid residue has been added, deleted or replaced by another amino acid residue, with the altered portion retaining substantially the same antigen-binding specificity as a non-altered portion.

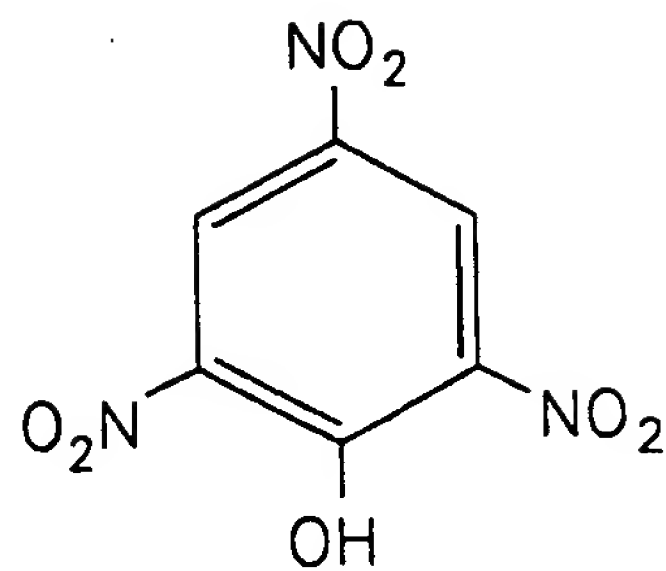
75. A protein according to Claim 74, being an antibody or a fragment of an
30 antibody.

76. A protein according to Claim 75, being a monoclonal antibody.
77. A protein having an antigen-binding portion having substantially the same antigen binding specificity of the construct according to any one of Claims 74-76.
78. A protein according to Claim 77, being an antibody or a fragment of an antibody.
- 5

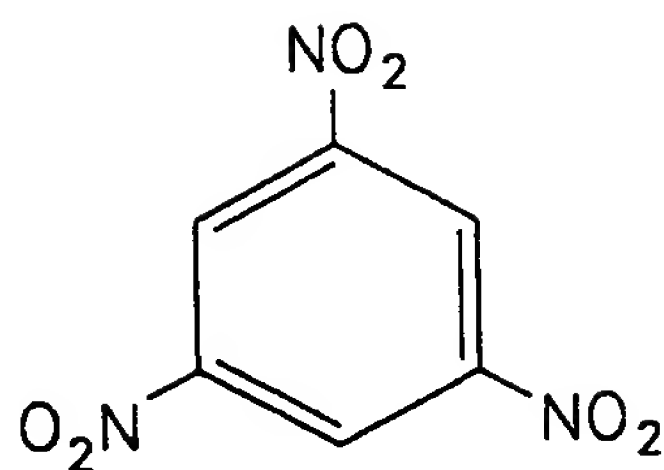
1/29



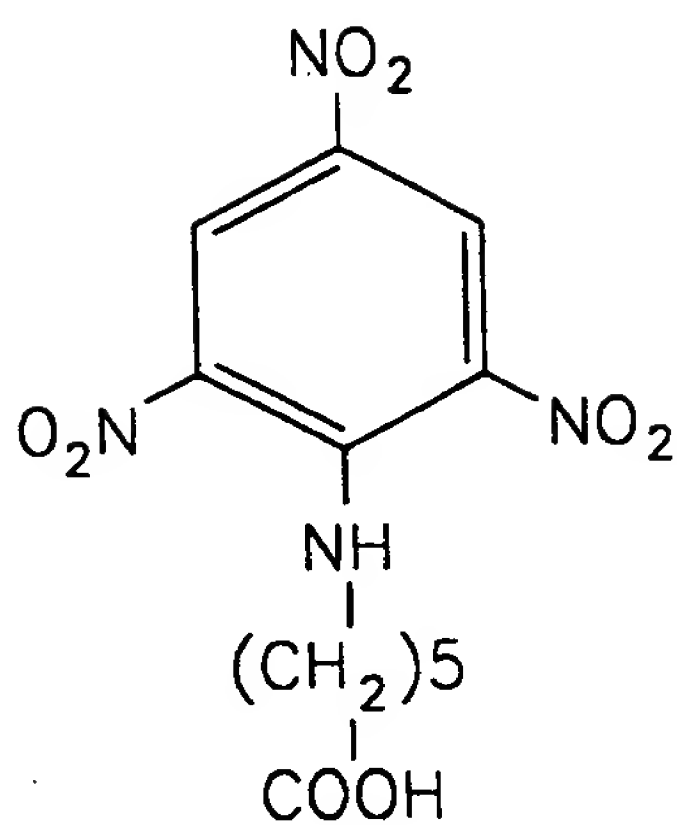
TNT



TNP



TNB



TNP-cap

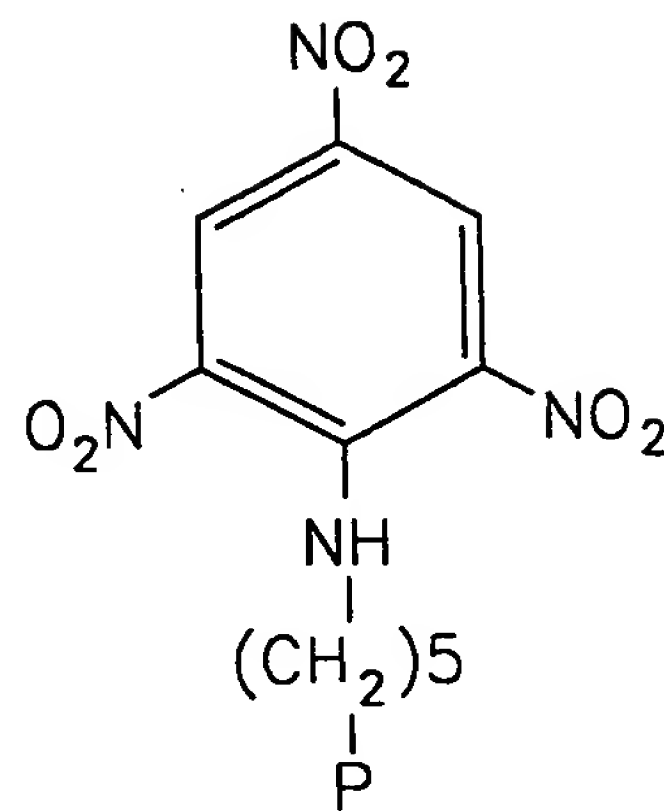
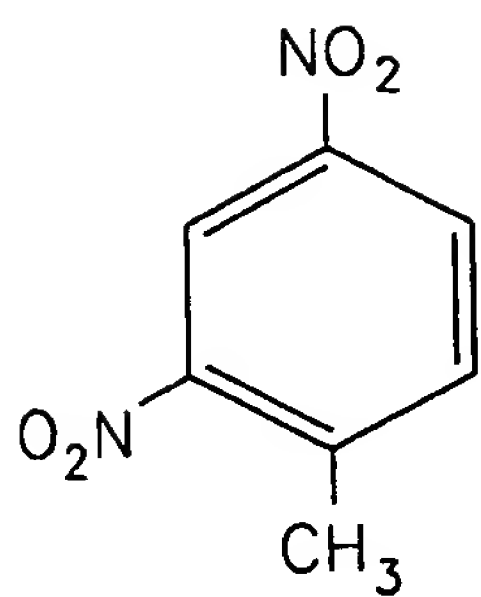
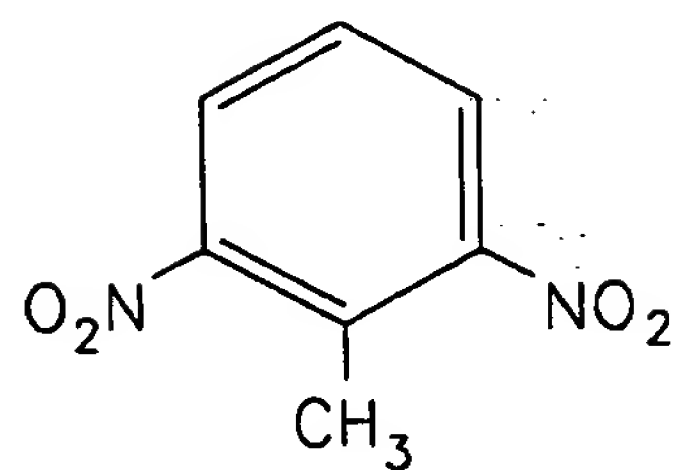


FIG.1A

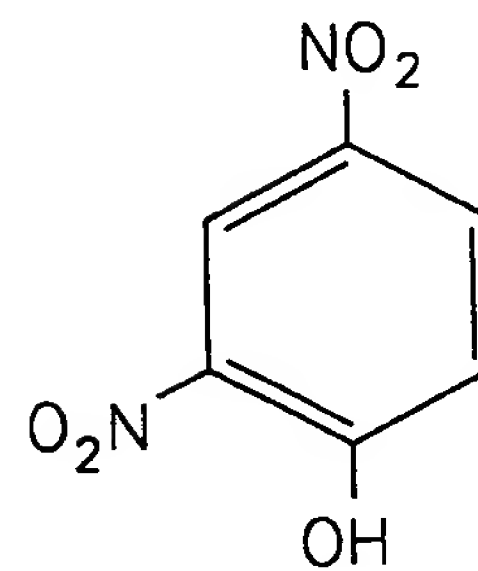
2/29



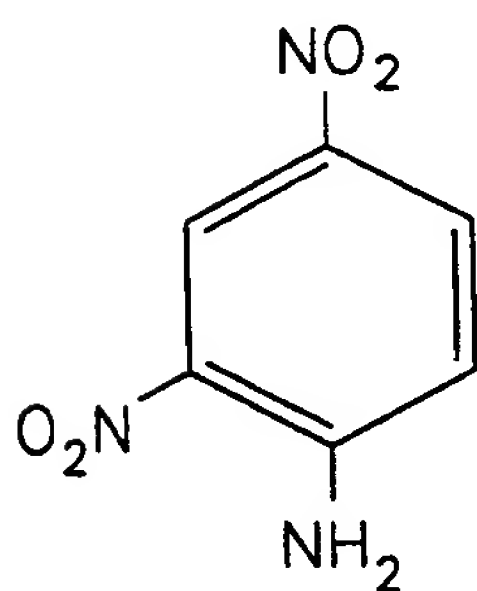
2,4-DNT



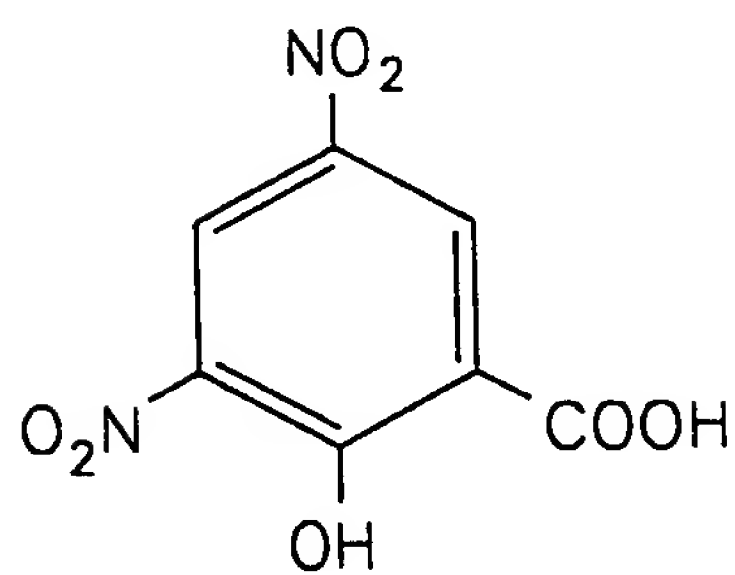
2,6-DNT



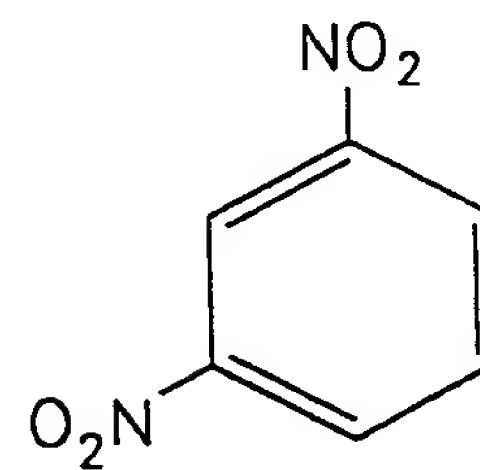
2,4-DNP



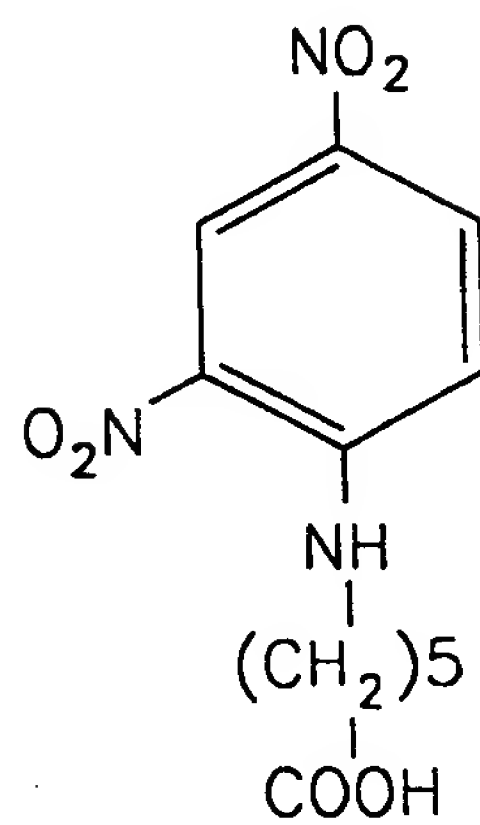
2,4-DNA



DNS



m-DNB



2,4-DNP-cap

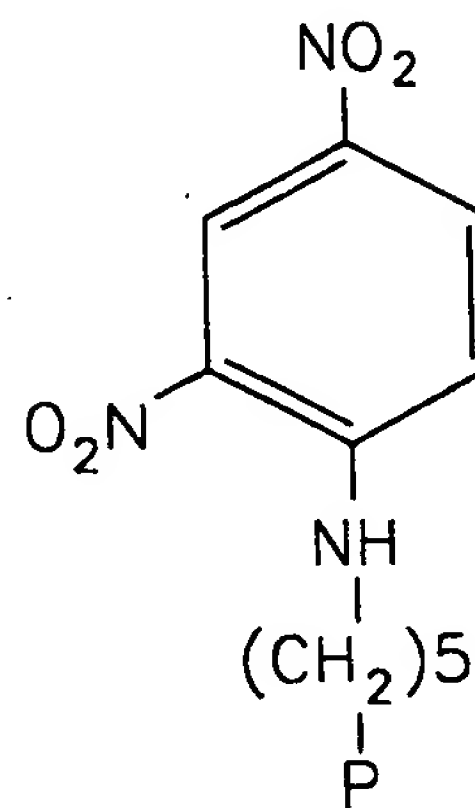


FIG.1B

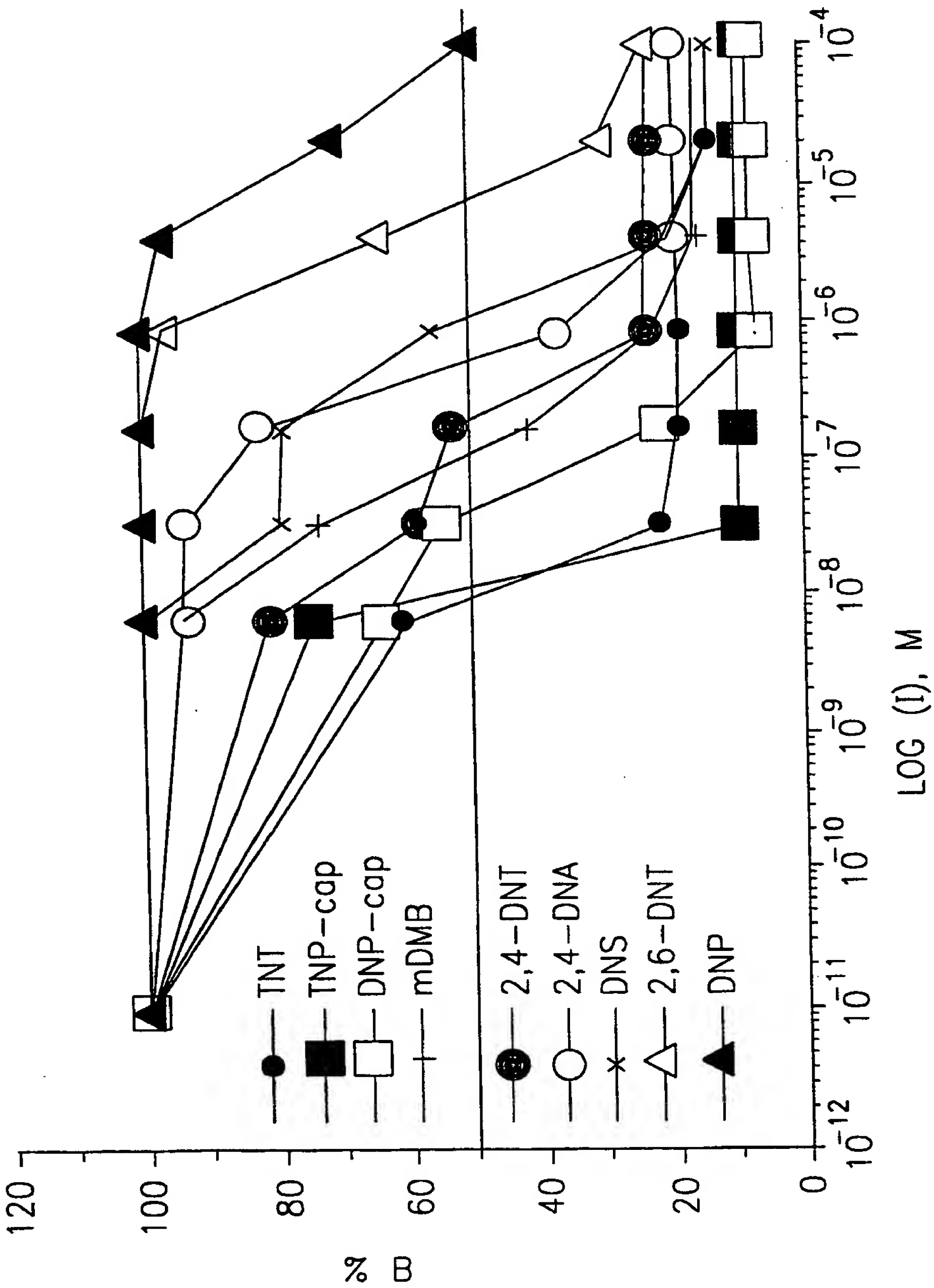


FIG.2A

4/29

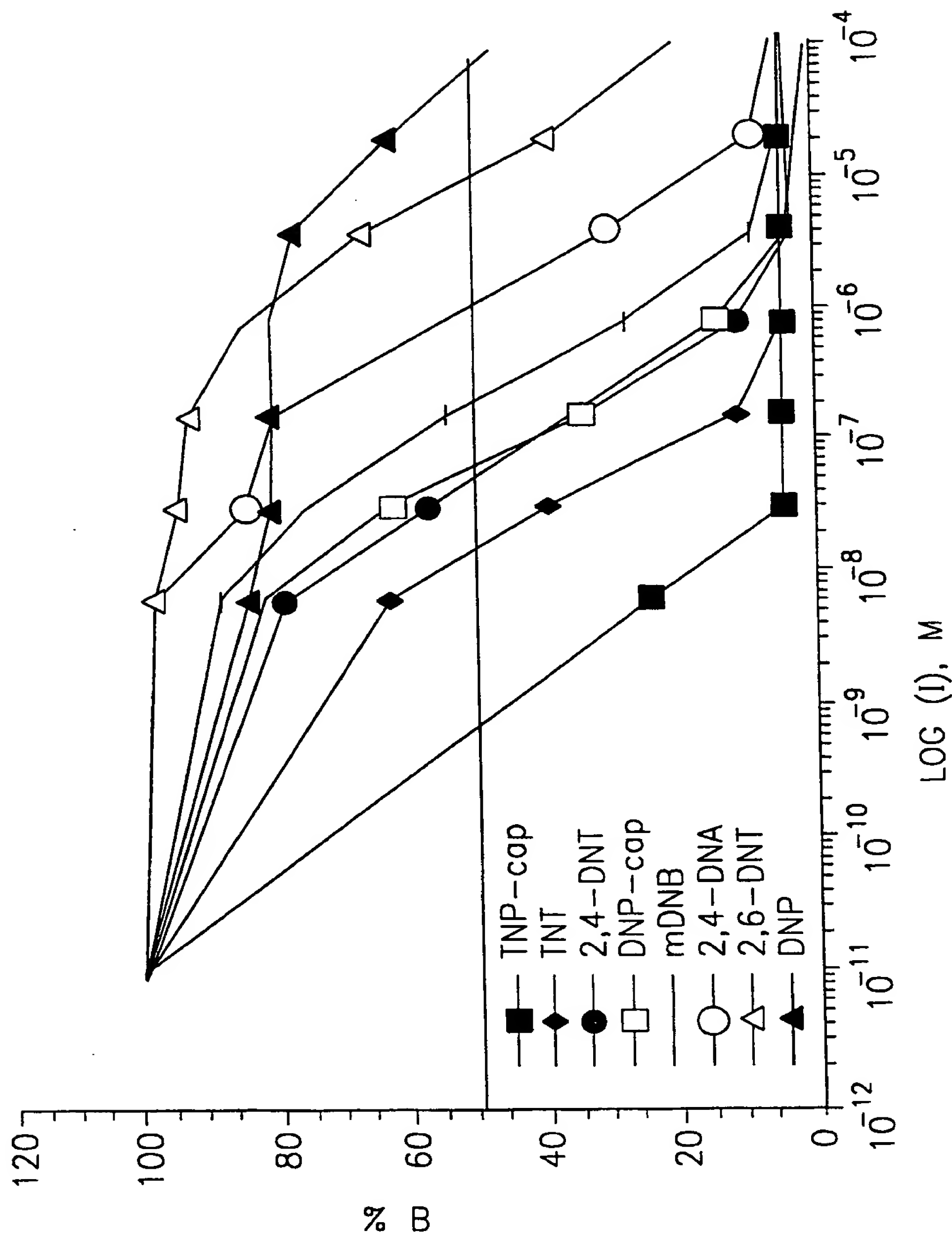


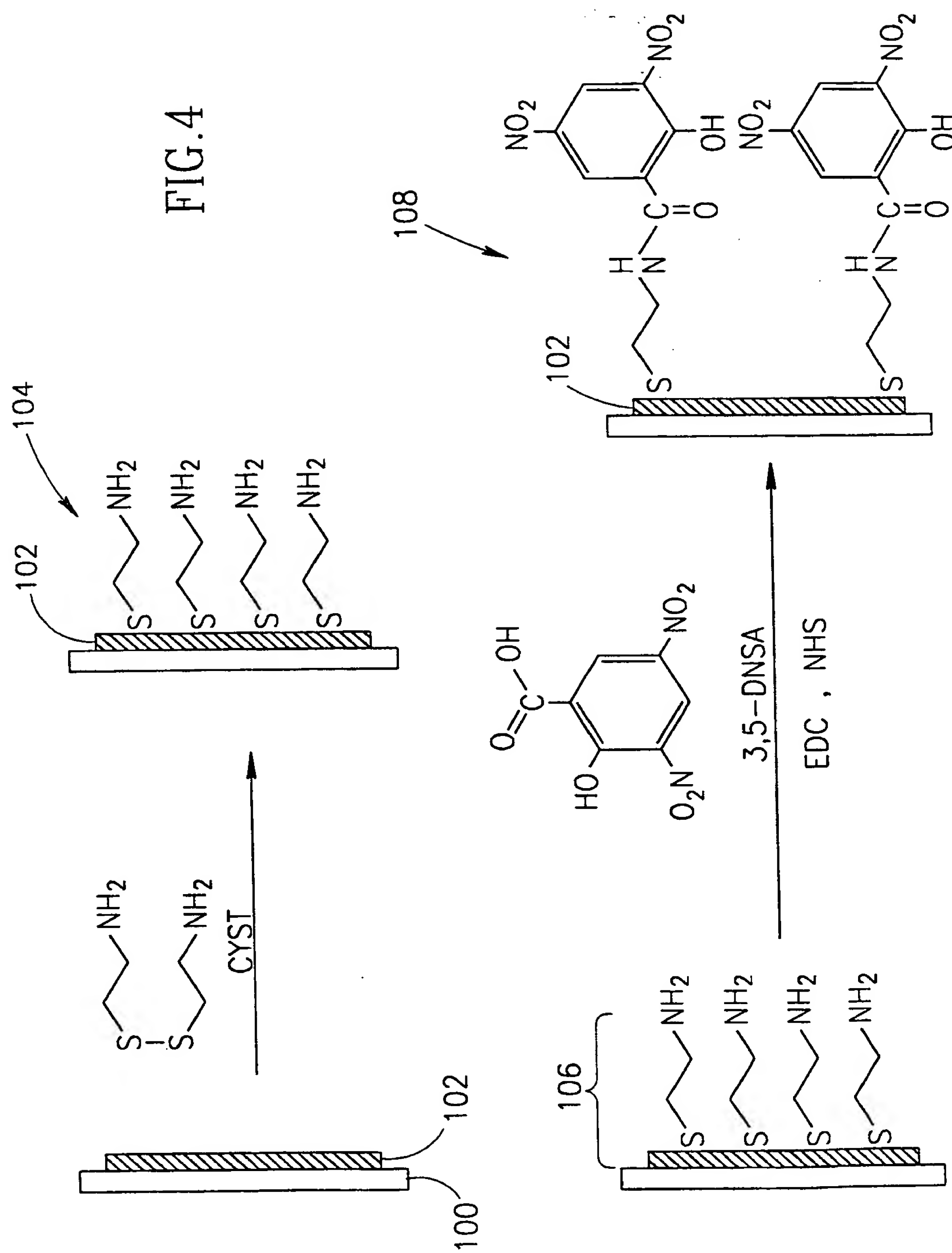
FIG.2B

6/29

1	TCT	AGA	GGA	GAT	ATC	GTT	ATG	ACC	CAG	TCT	CCA	TCC	TCC	CTG	AGT	GTG	TCA	GCA	GGA	GAG	60
	S	R	G	D	I	V	M	T	Q	S	P	S	S	L	S	V	S	A	G	E	
61	AAG	GTC	ACT	ATG	AGC	TGC	AAG	TCC	AGT	CAG	AGT	CTG	TTA	AAC	AGT	AGA	AAT	CAA	AAG	AAC	120
	K	V	T	M	S	C	K	S	S	Q	S	L	L	N	S	R	N	Q	K	N	
121	TAC	TTG	GCC	TGG	TAC	CAG	CAG	AAA	CCA	GGA	CAG	CCT	CCT	AAA	CTT	TTG	ATC	TAC	GGG	GTA	180
	Y	L	A	W	Y	Q	Q	K	P	G	Q	P	P	K	L	L	I	Y	G	V	
181	TTT	ATT	AGG	GAT	TCT	GGG	GTC	CCT	GAT	CGC	TTC	ACA	GGC	AGT	GGA	TCT	GGA	ACC	GAT	TTC	240
	F	I	R	D	S	G	V	P	D	R	F	T	G	S	G	S	G	T	D	F	
241	ACT	CIT	ACC	ATC	AGC	AGT	GTG	CAG	GCT	GAA	GAC	CTG	GCA	GTT	TAT	TAC	TGT	CAG	AAT	GAT	300
	T	L	T	I	S	S	V	Q	A	E	D	L	A	V	Y	Y	C	Q	T	D	
301	CAT	ATT	TAT	CCG	TAC	ACG	TTC	GGA	GGG	GGG	ACC	AAG	CTG	GAA	ATA	AAA					
	H	I	Y	P	Y	T	F	G	G	G	T	K	L	E	I	K					

FIG.3B

FIG. 4



8/29

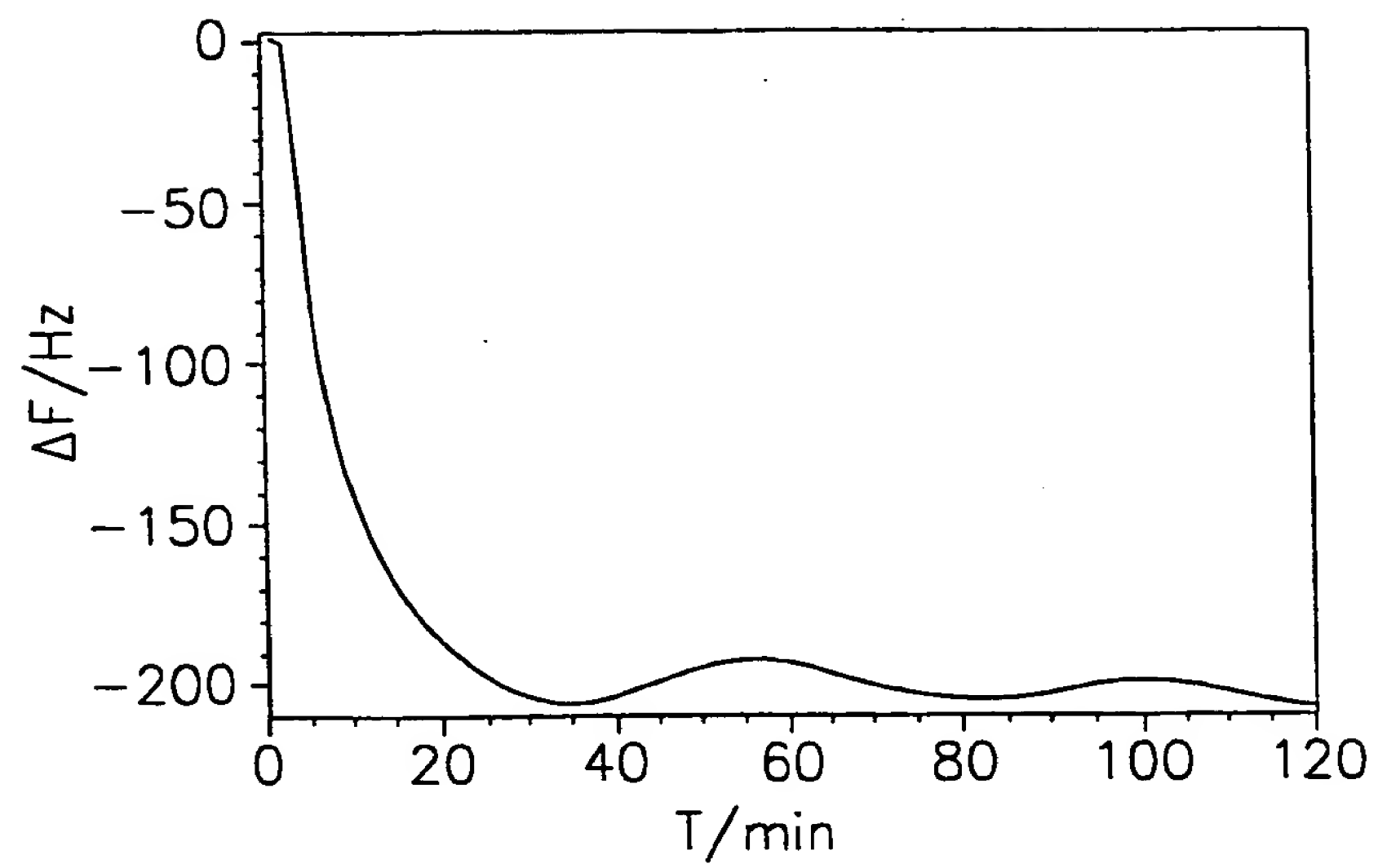


FIG.5

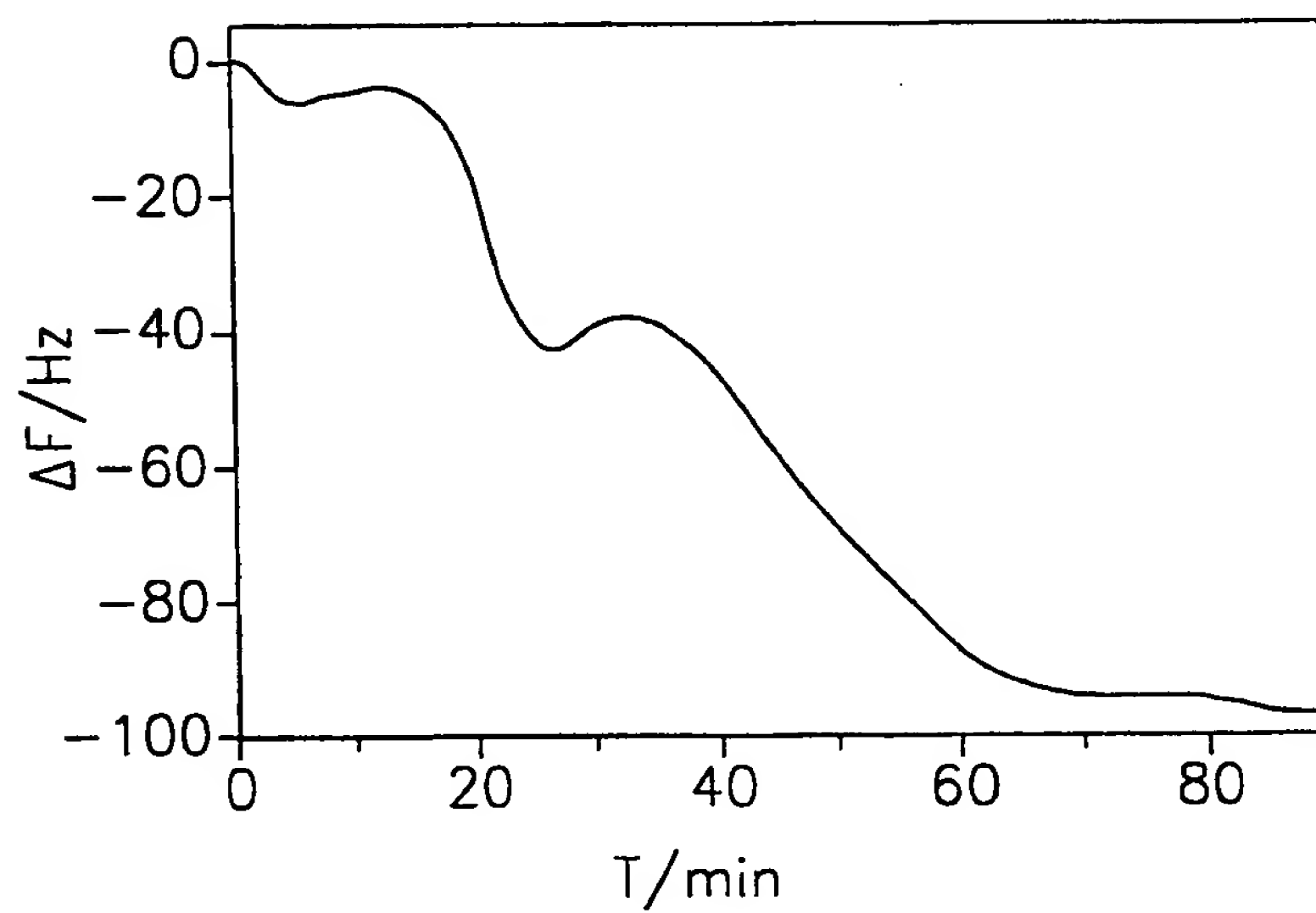


FIG.6

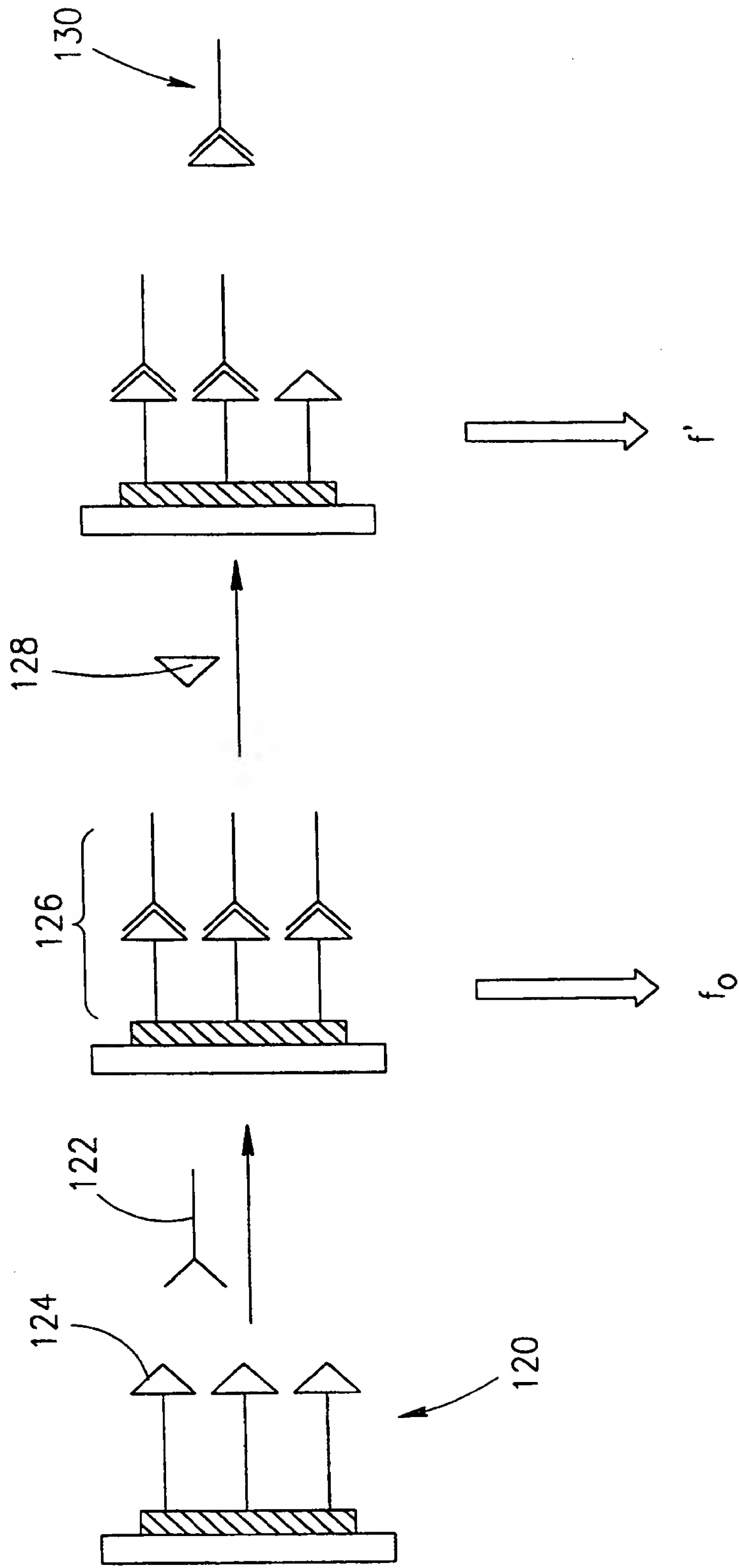


FIG.7

10/29

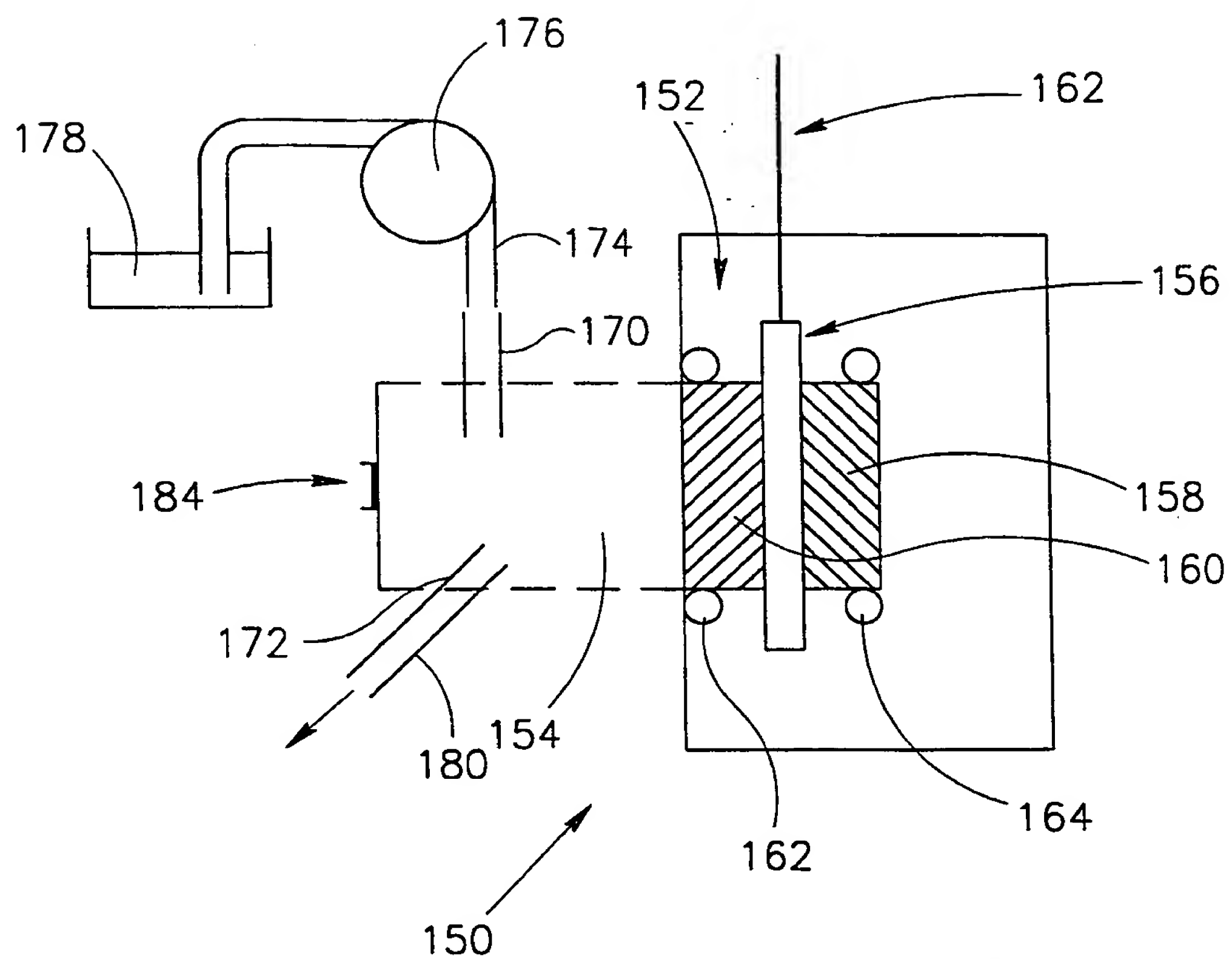


FIG. 8

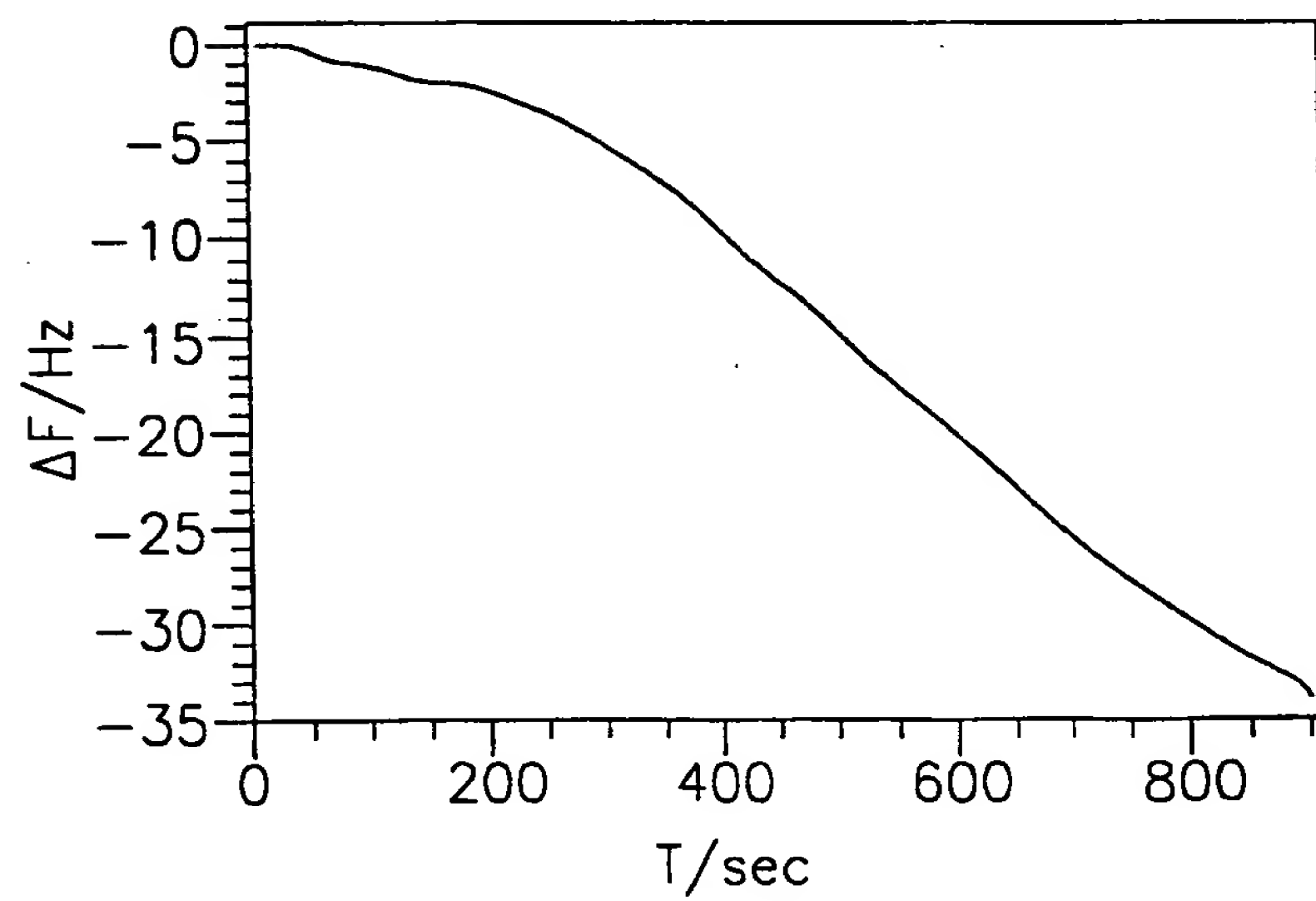


FIG. 9

11/29

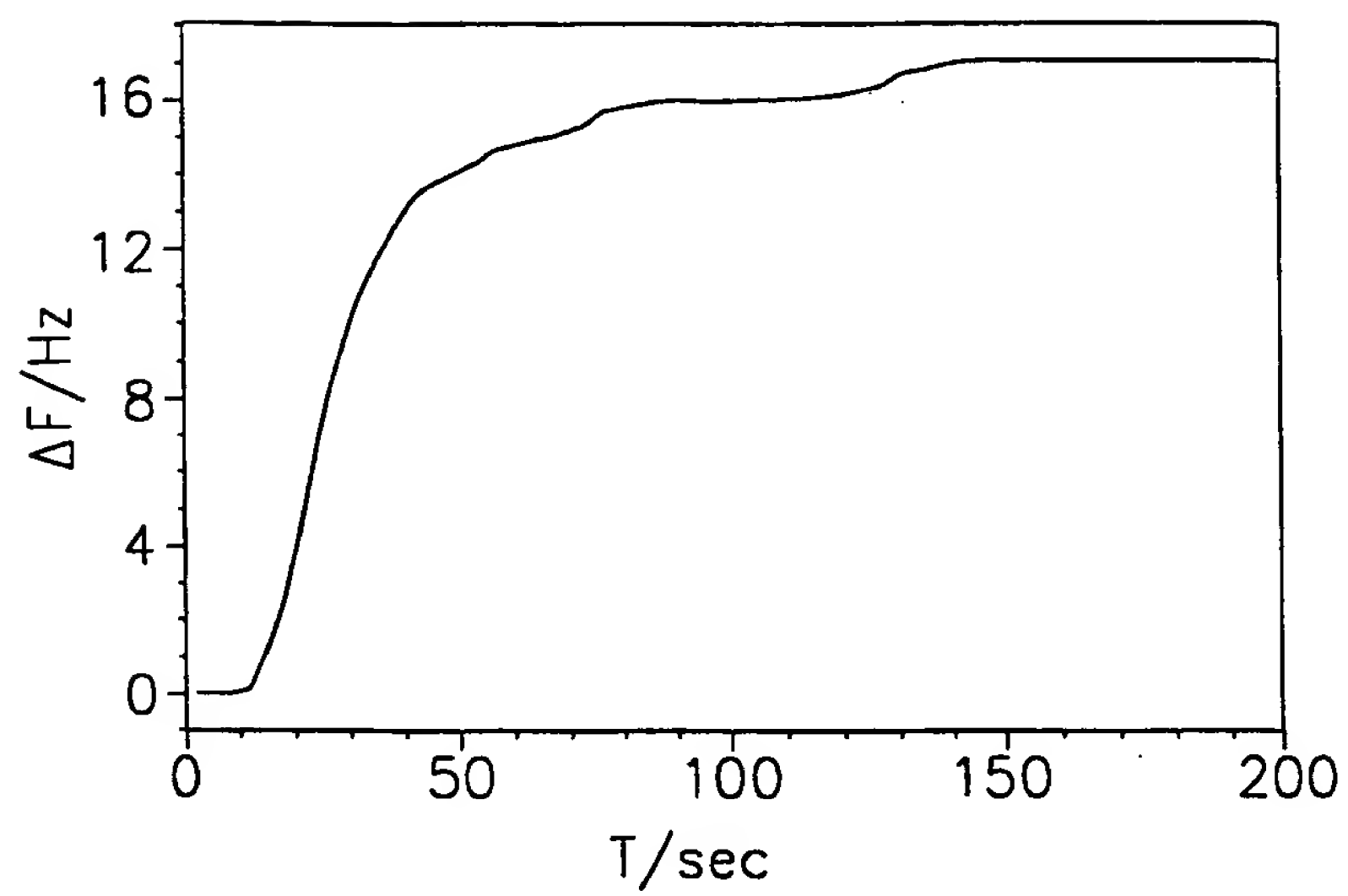


FIG.10

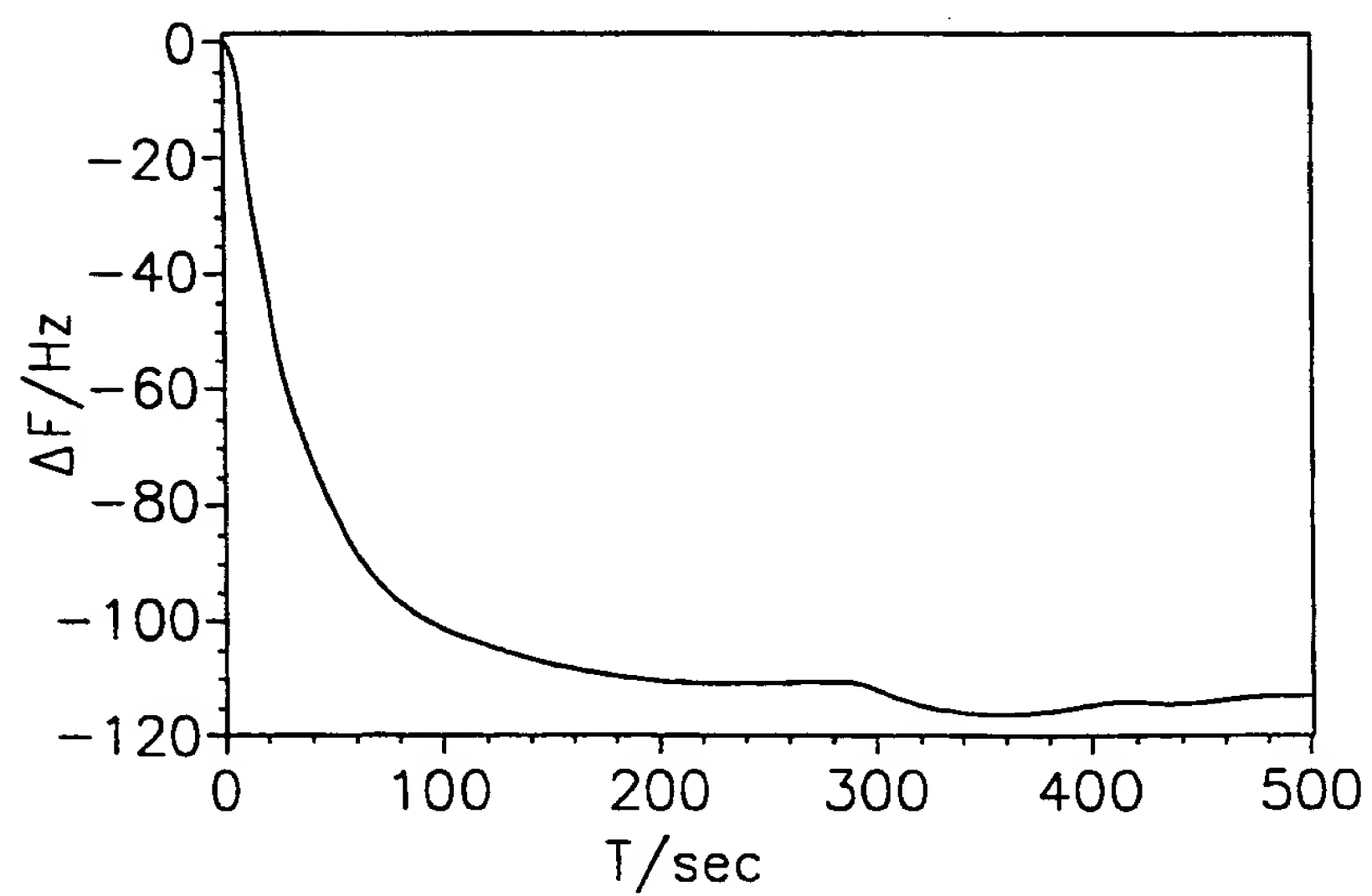


FIG.11

12/29

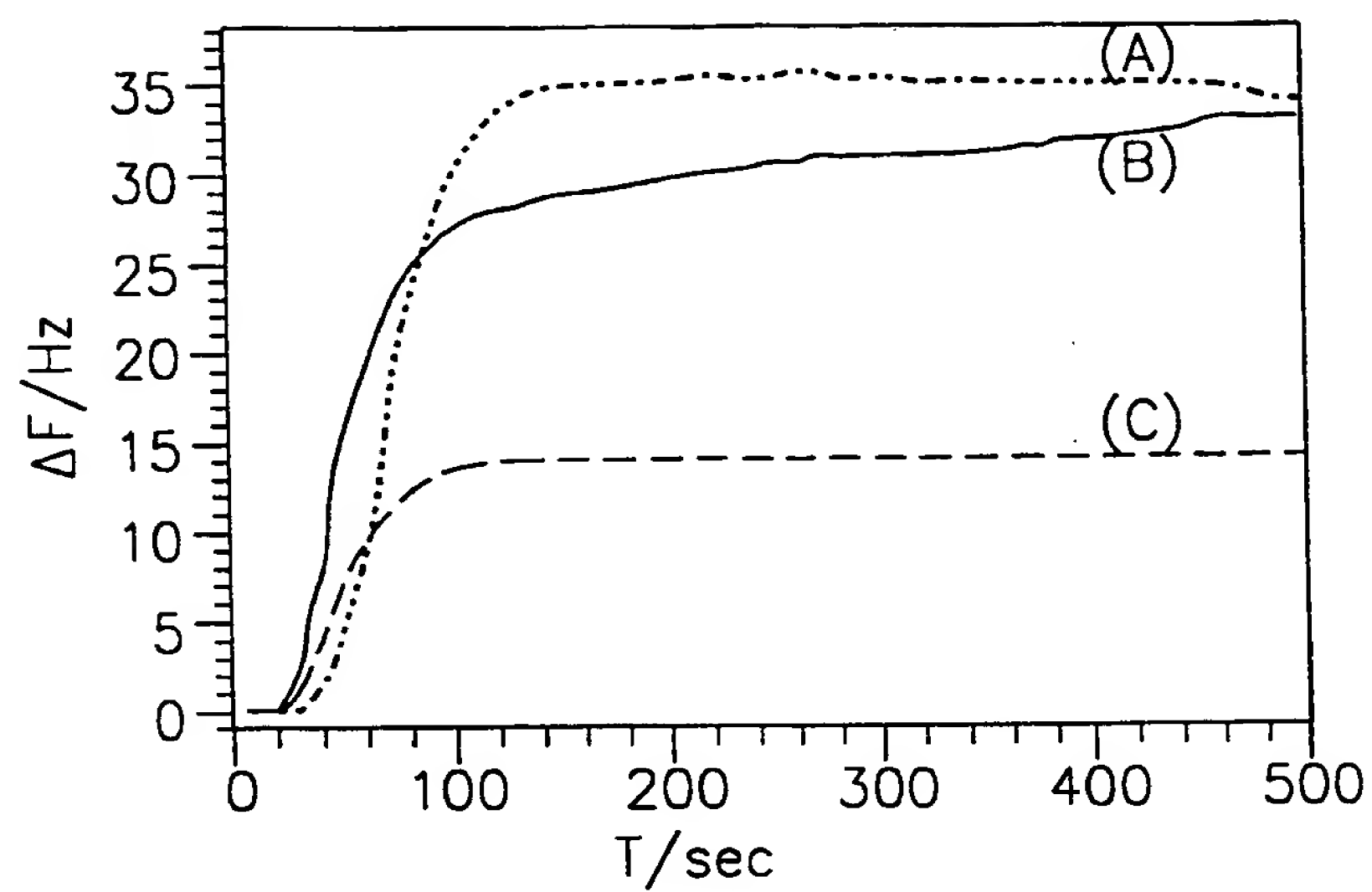


FIG.12

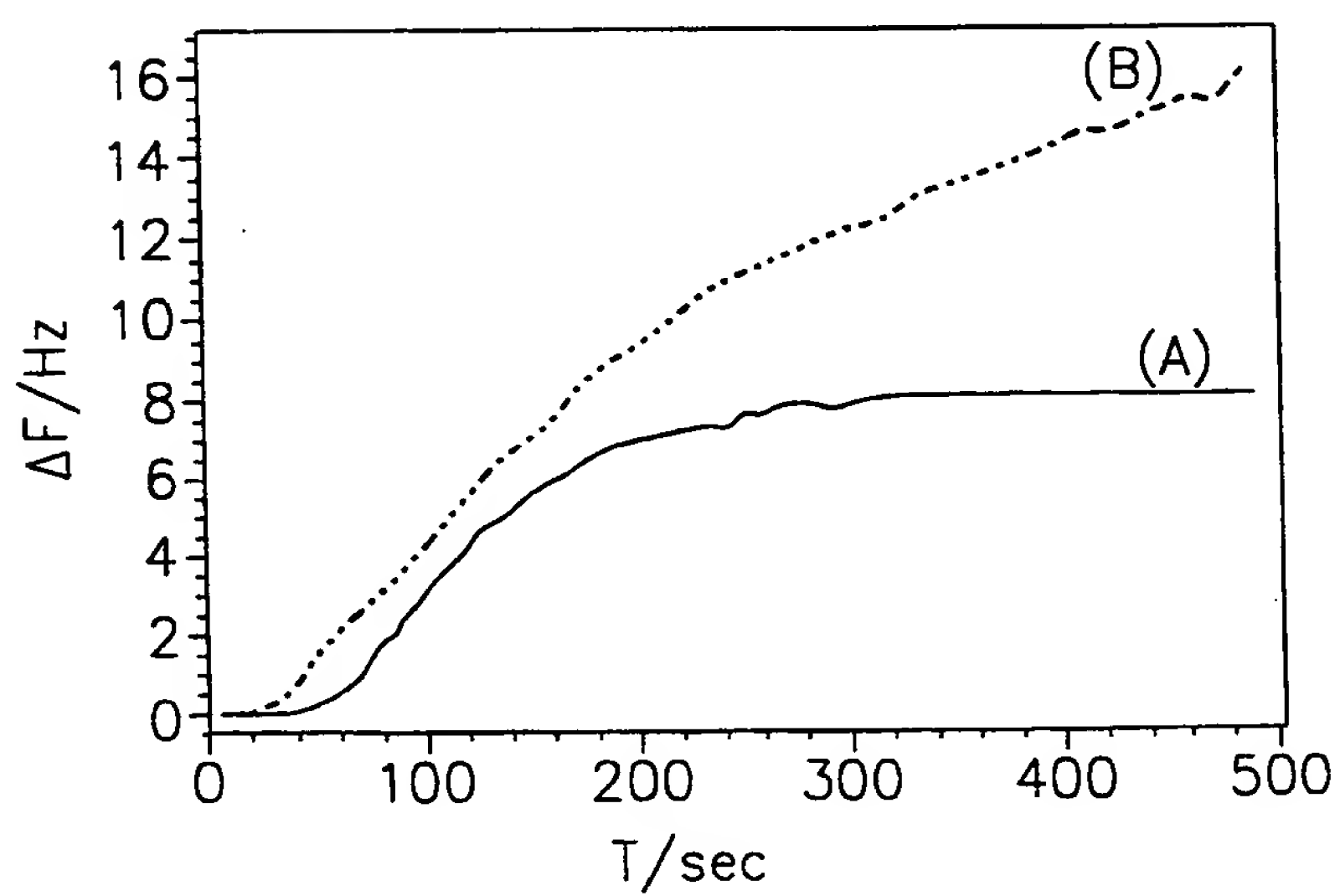


FIG.13

13/29

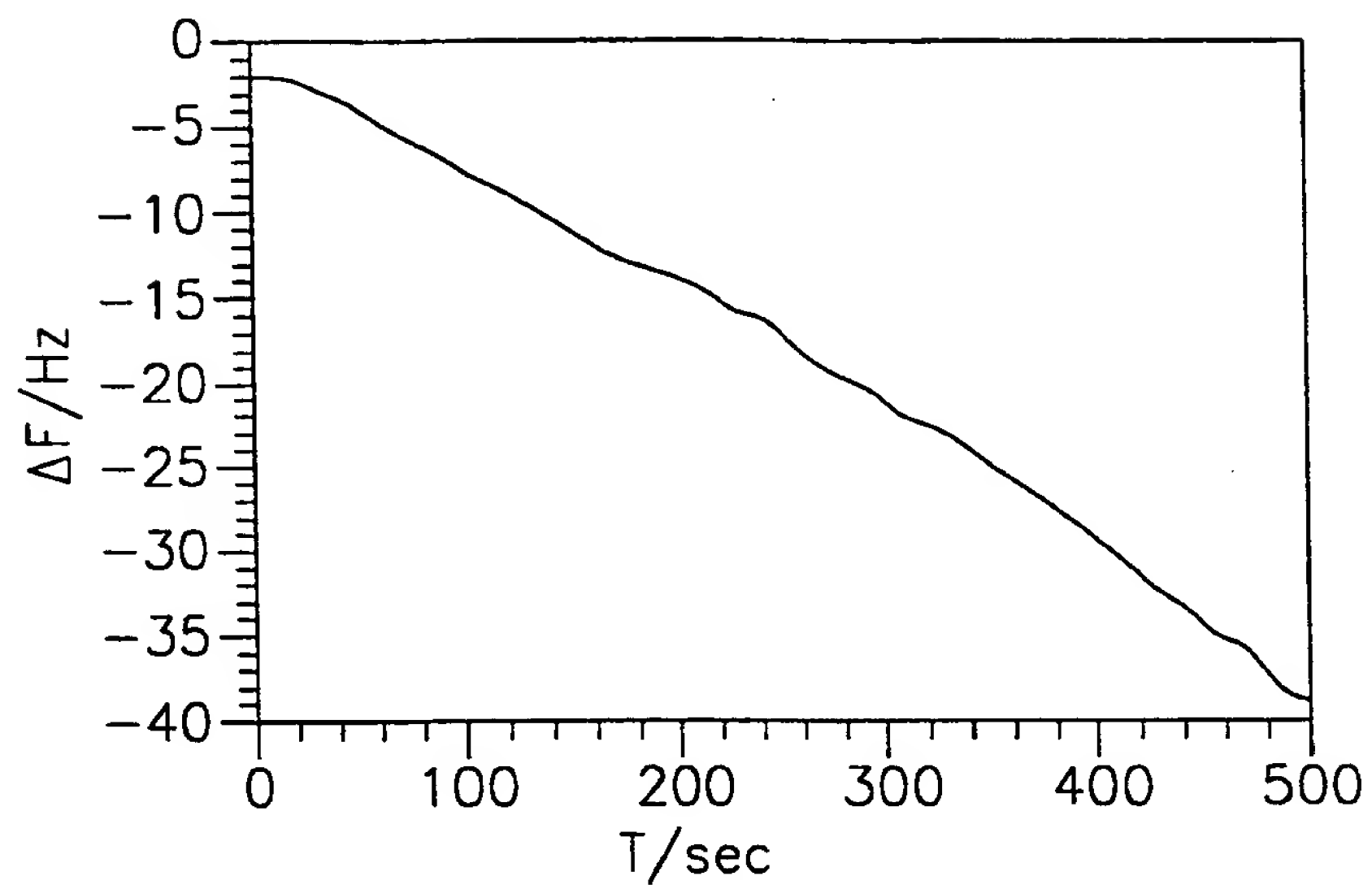


FIG.14

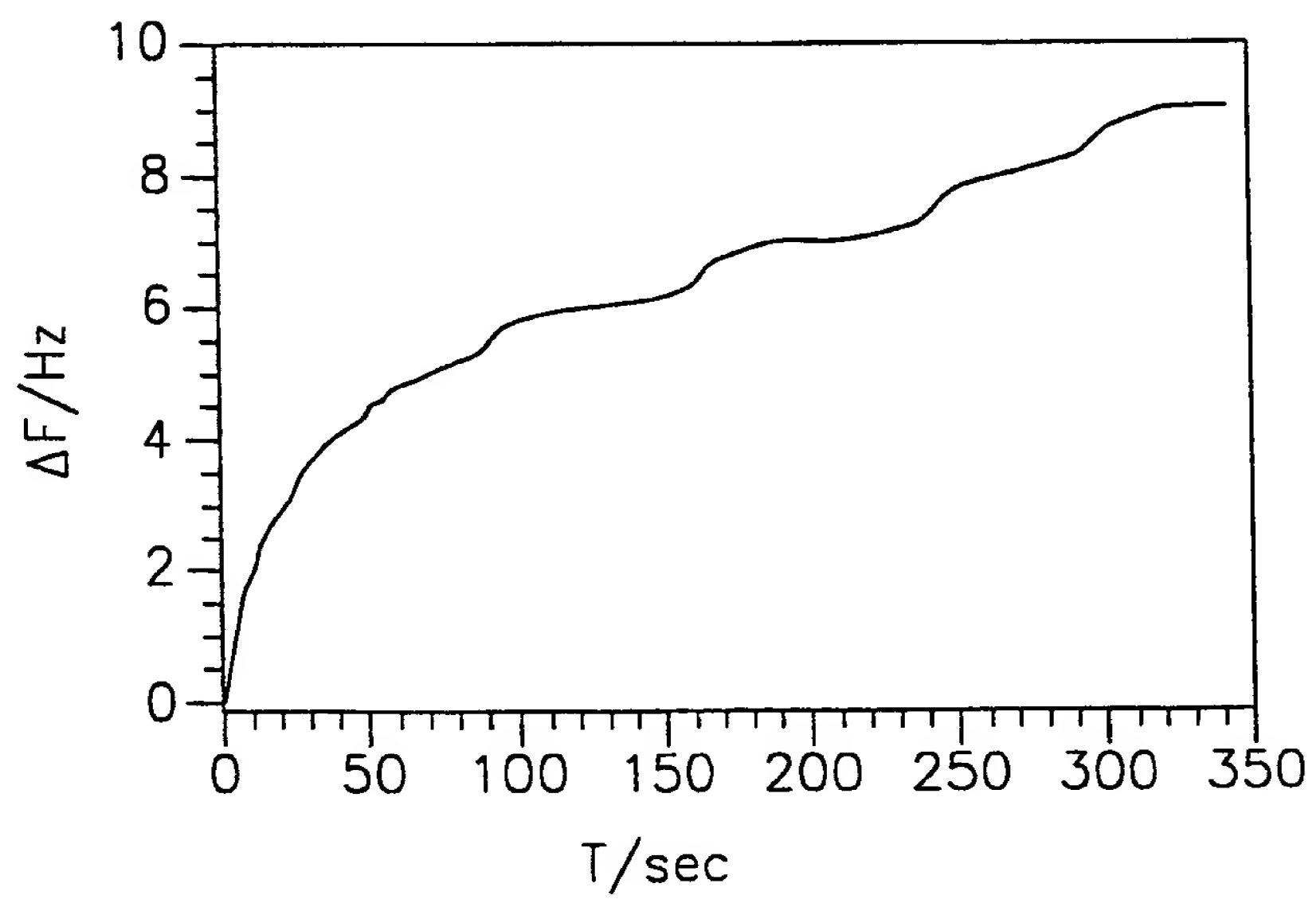


FIG.15

14/29

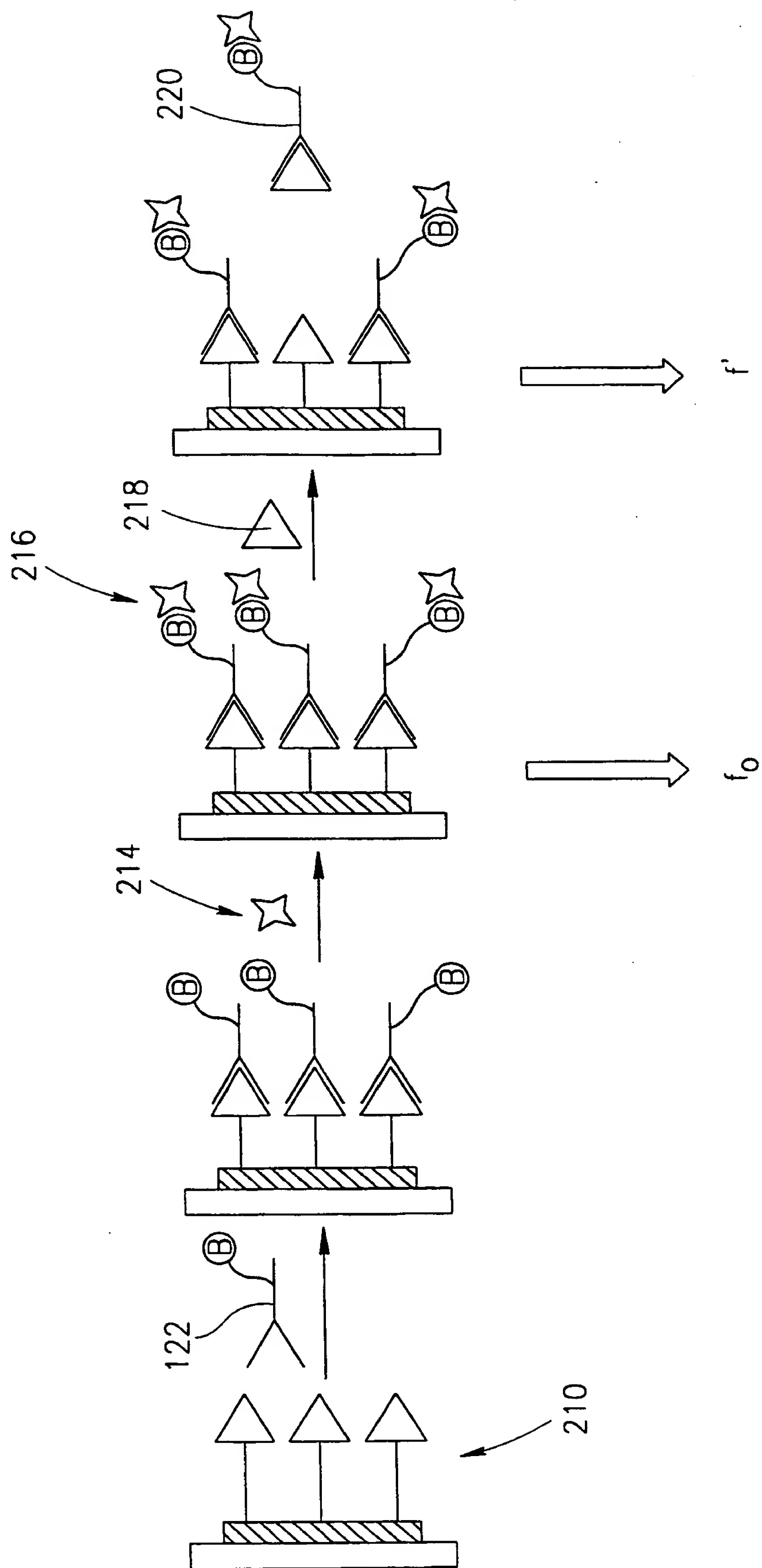


FIG.16

15/29

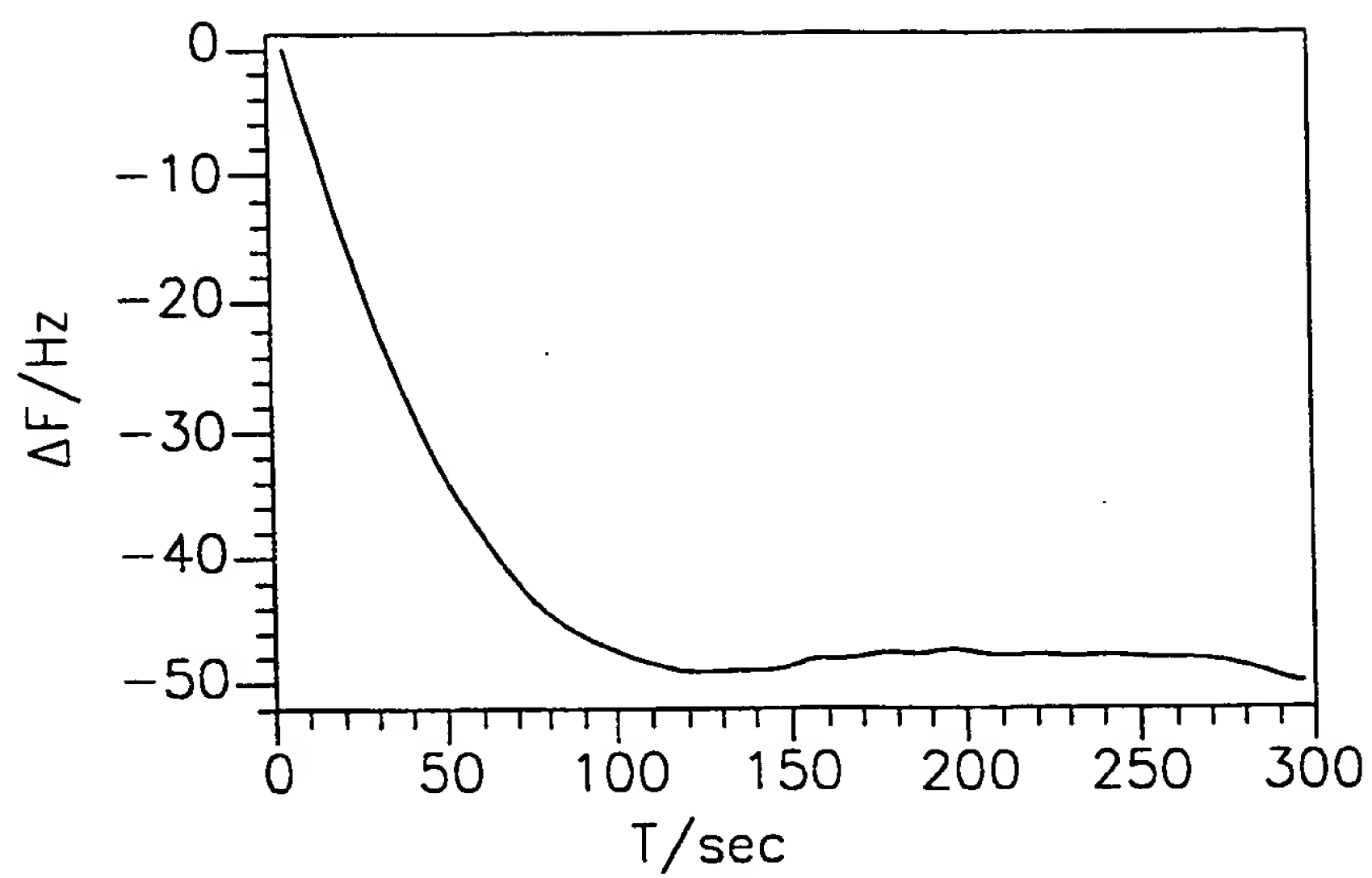


FIG.17

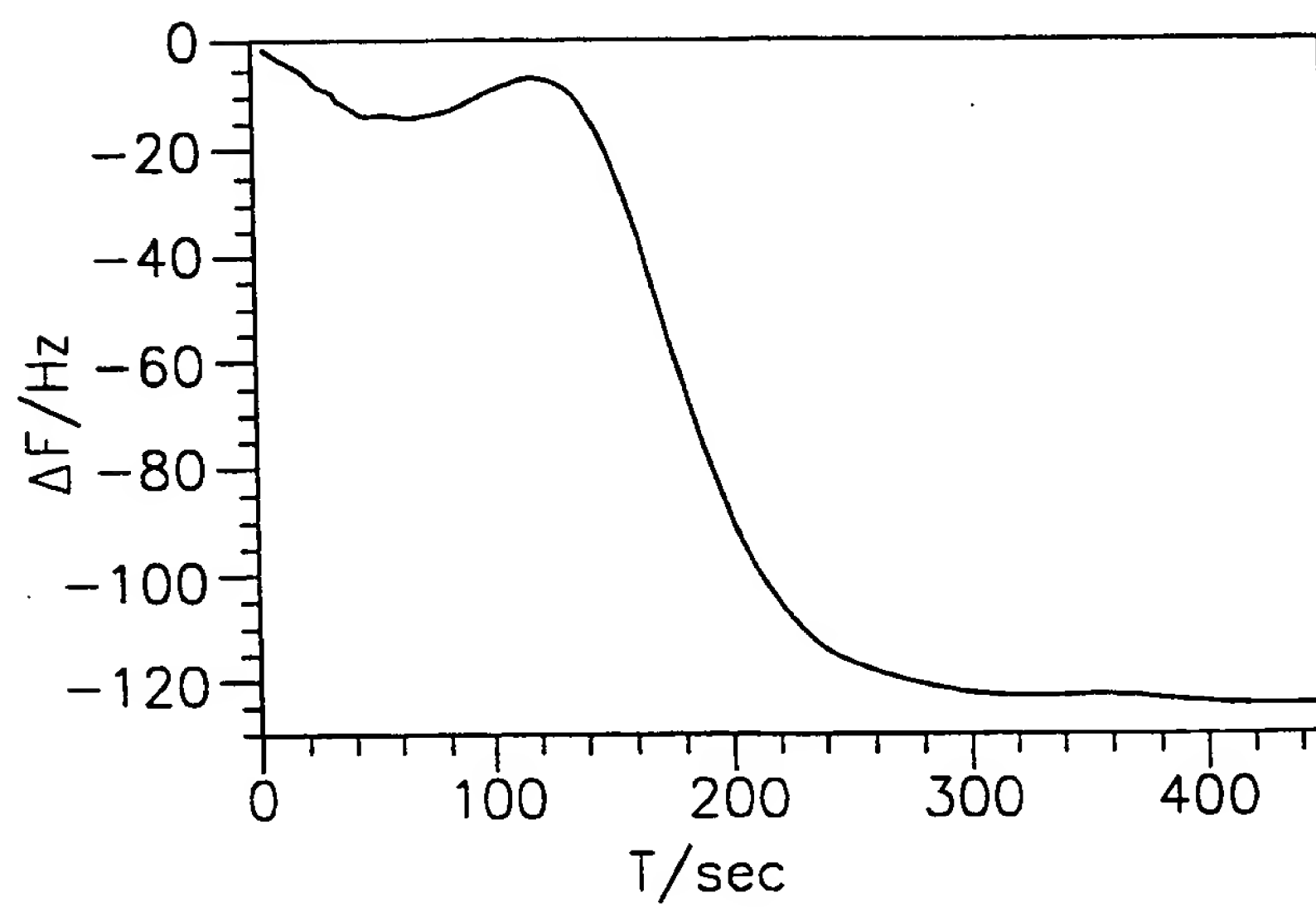


FIG.18

16/29

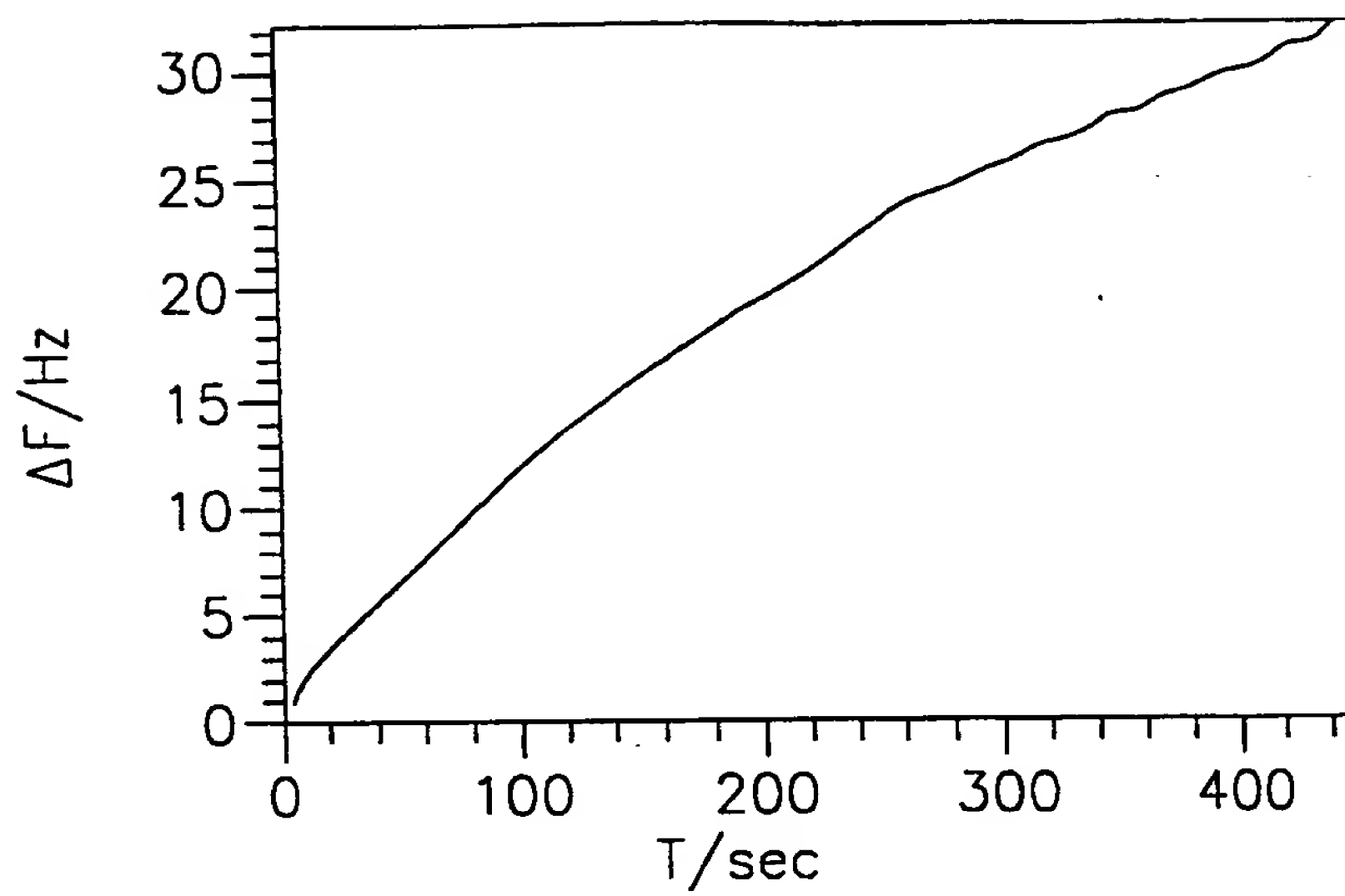


FIG.19

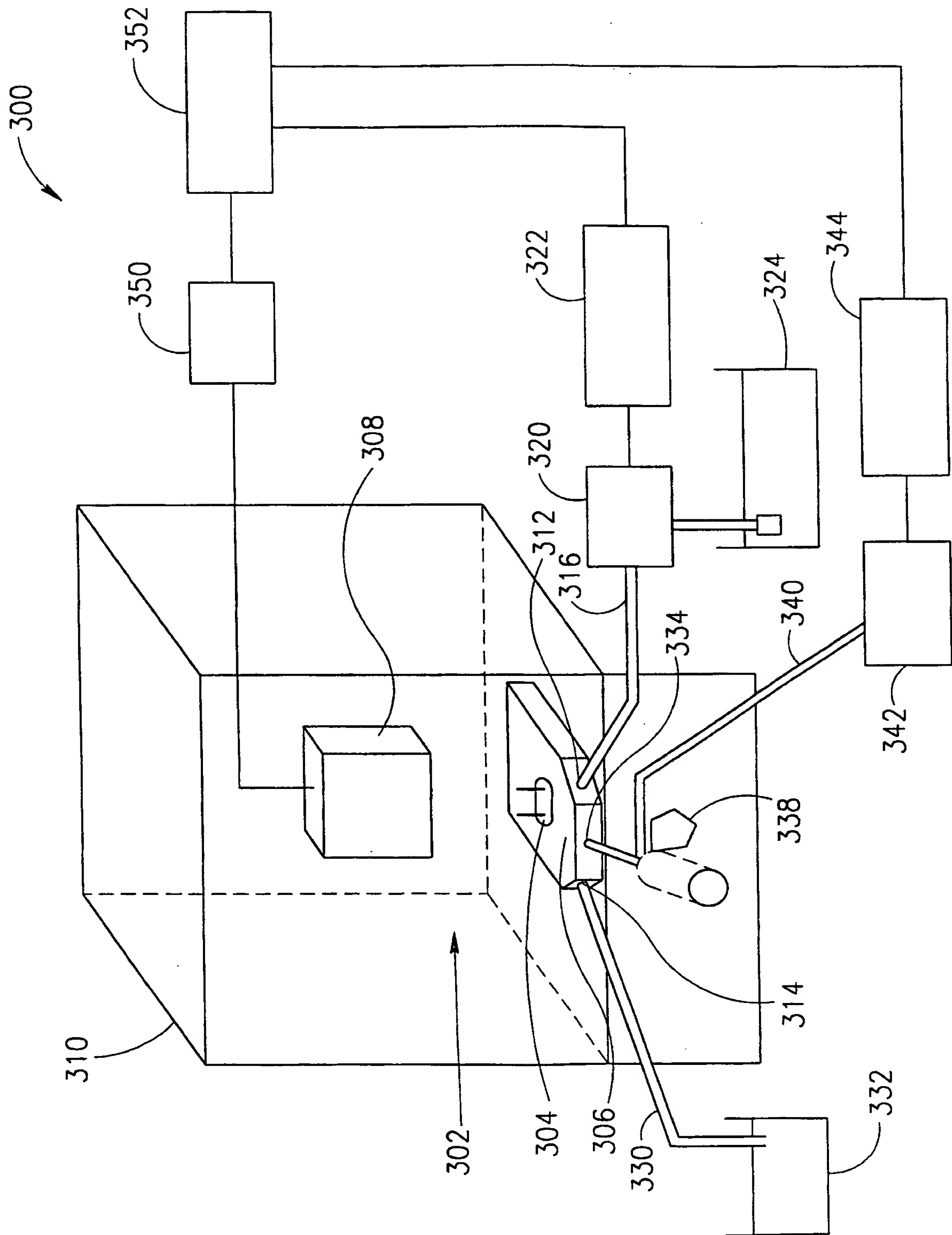


FIG. 20

18/29

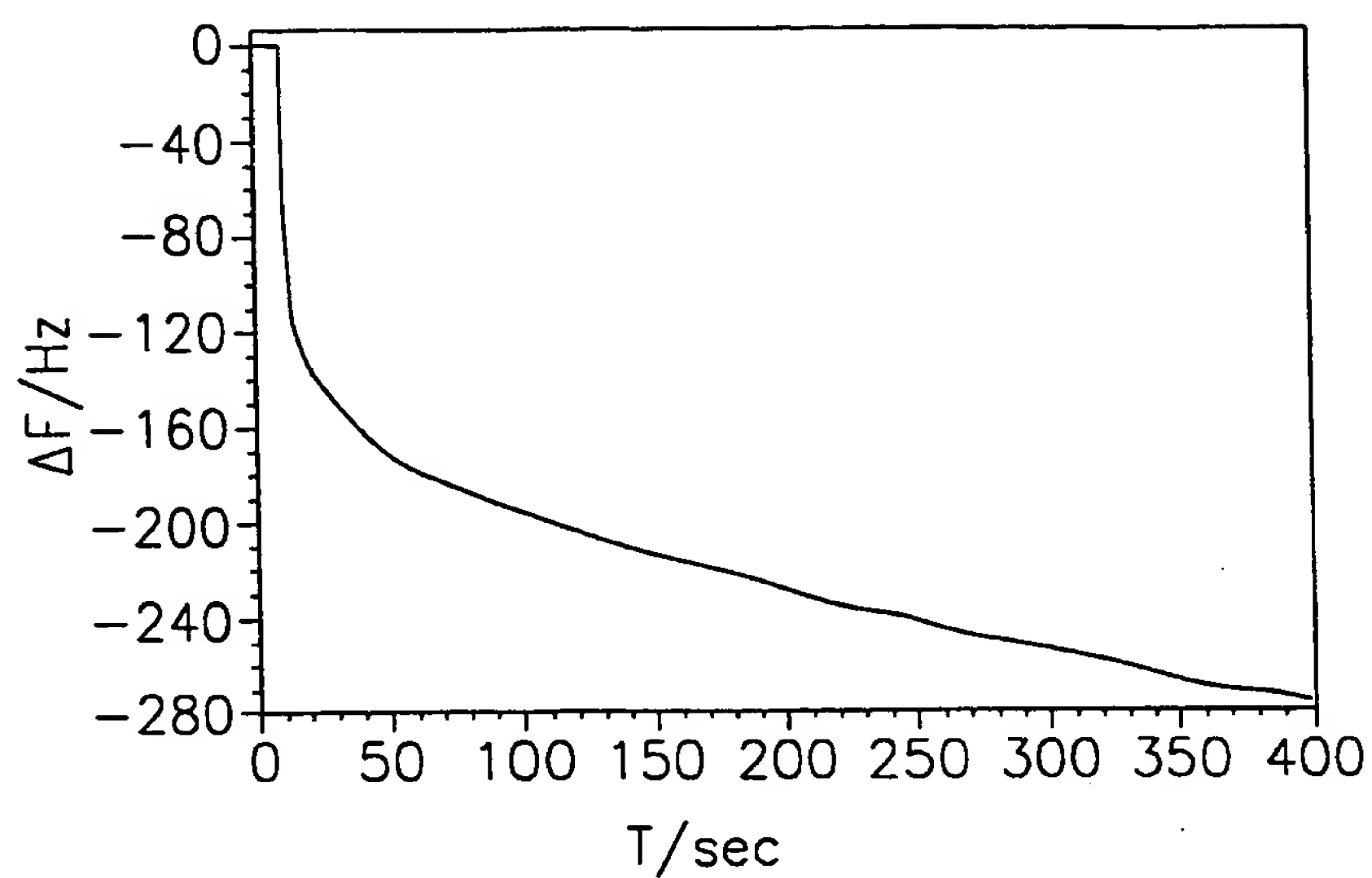


FIG. 21

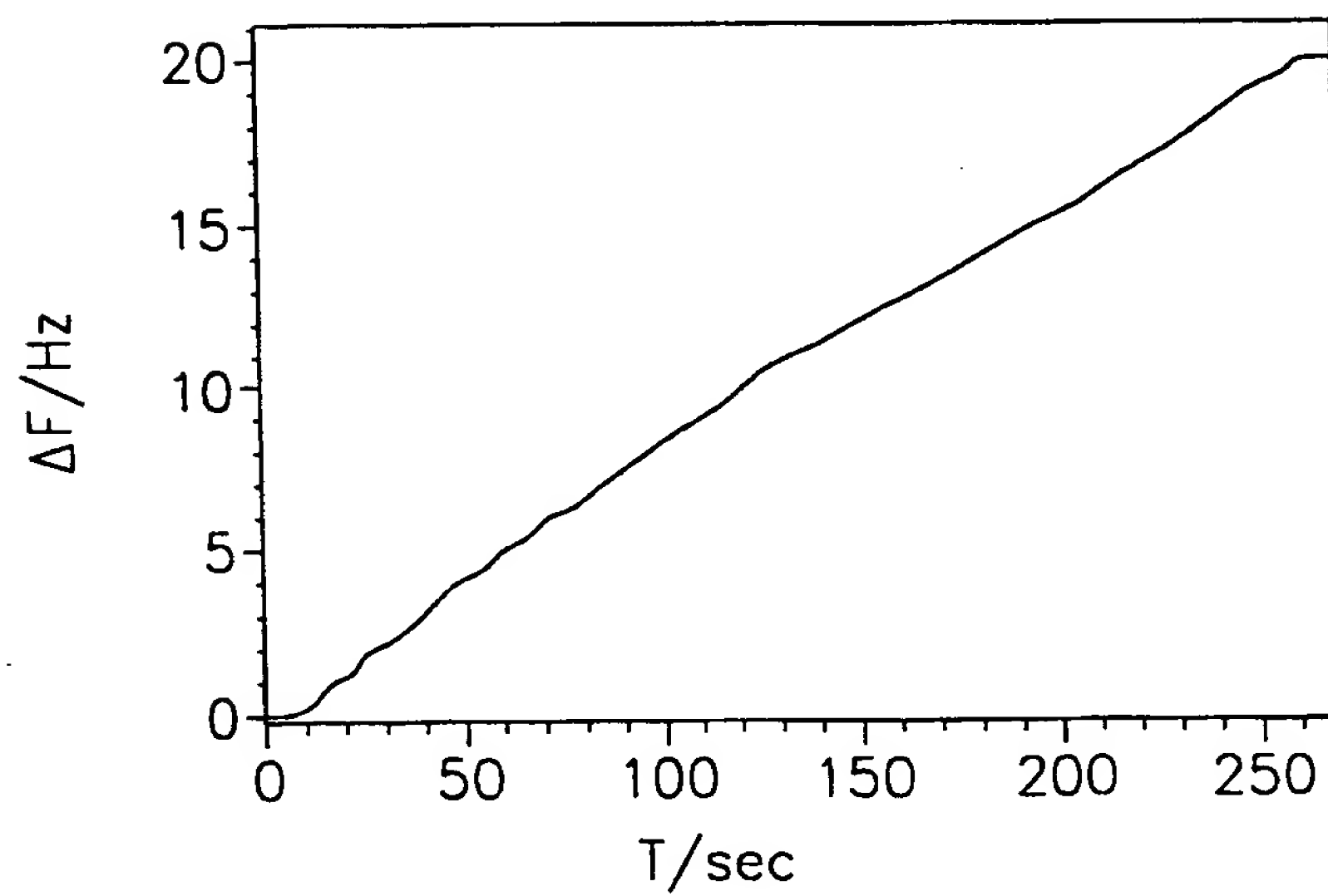


FIG. 22

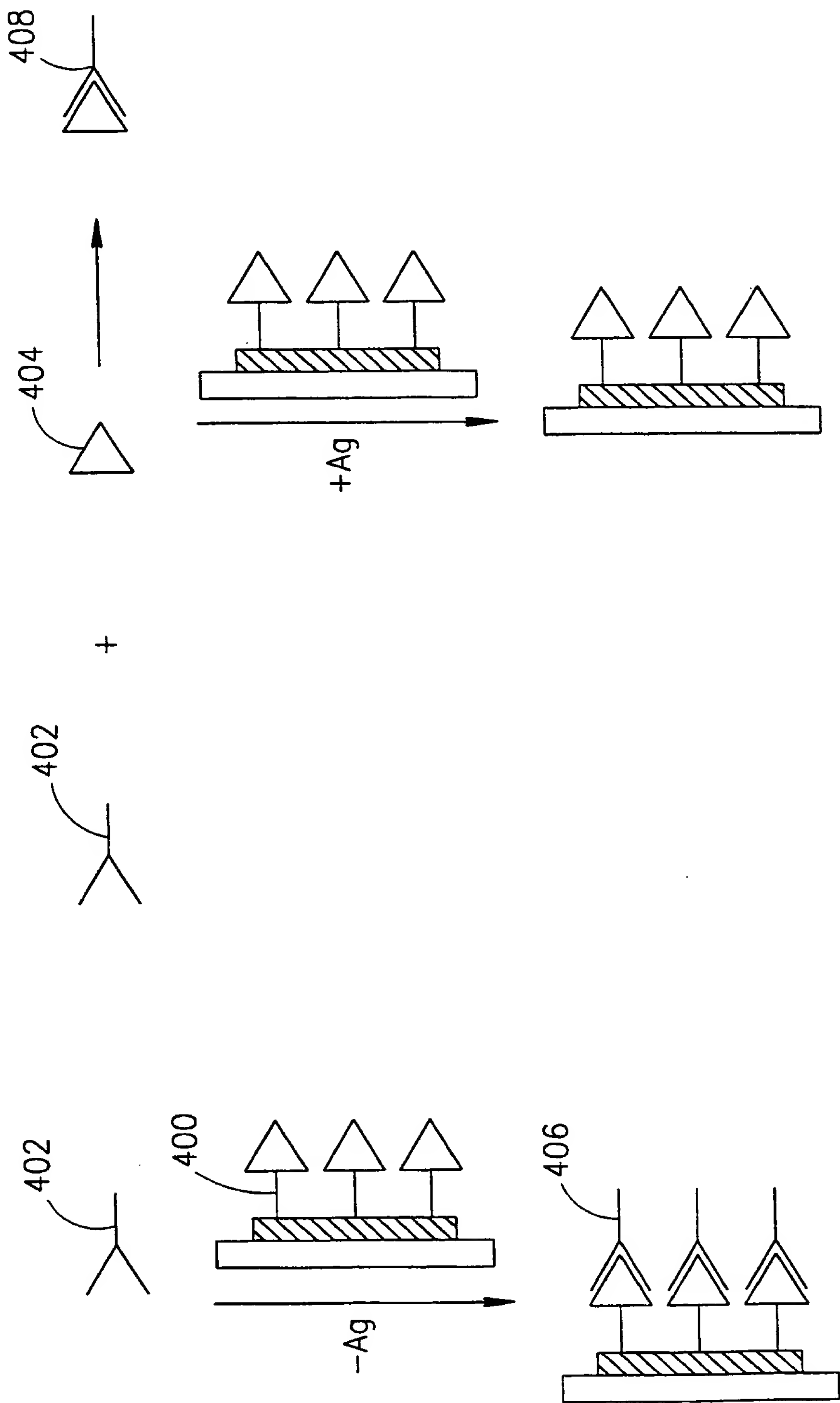


FIG.23

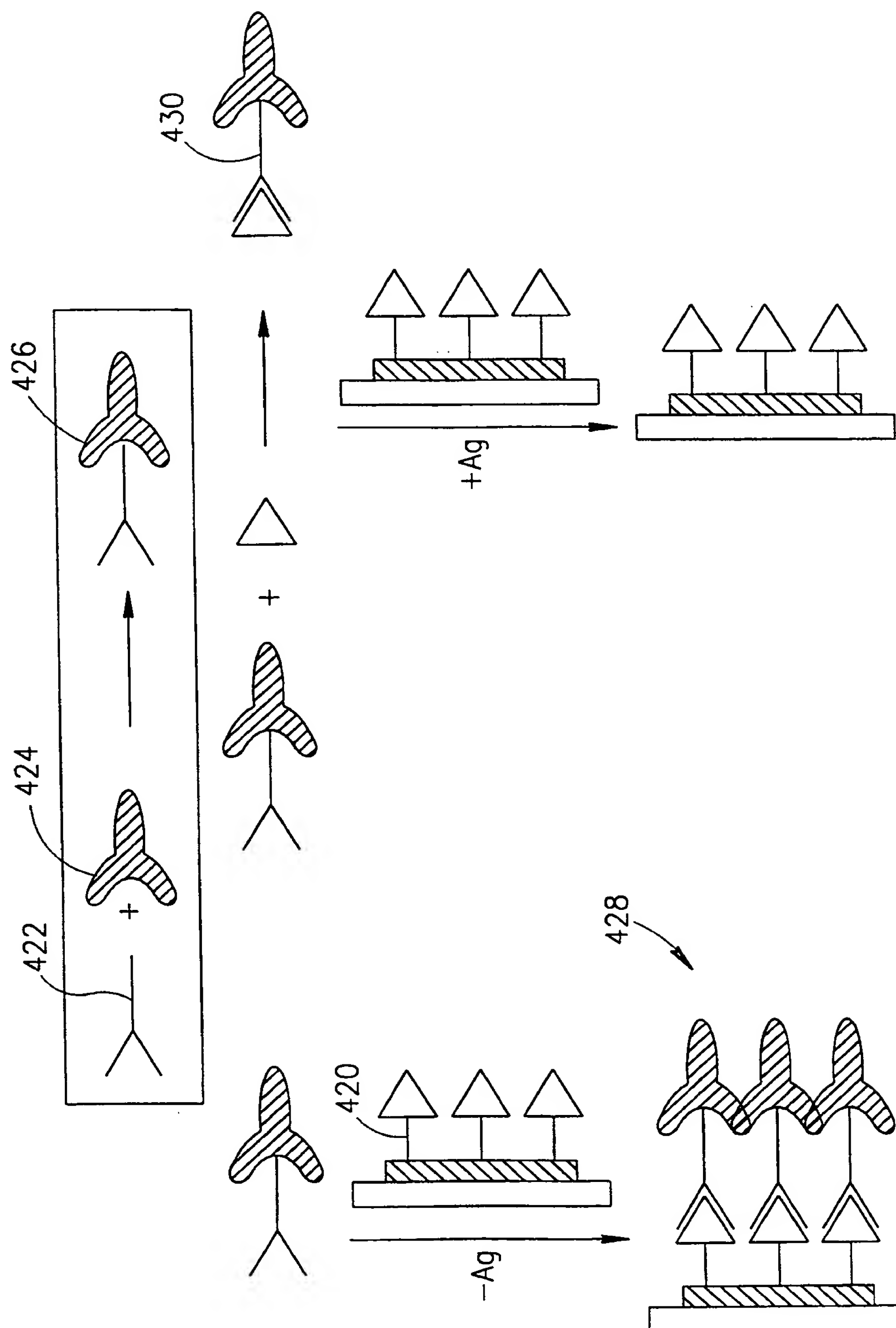


FIG.24

21/29

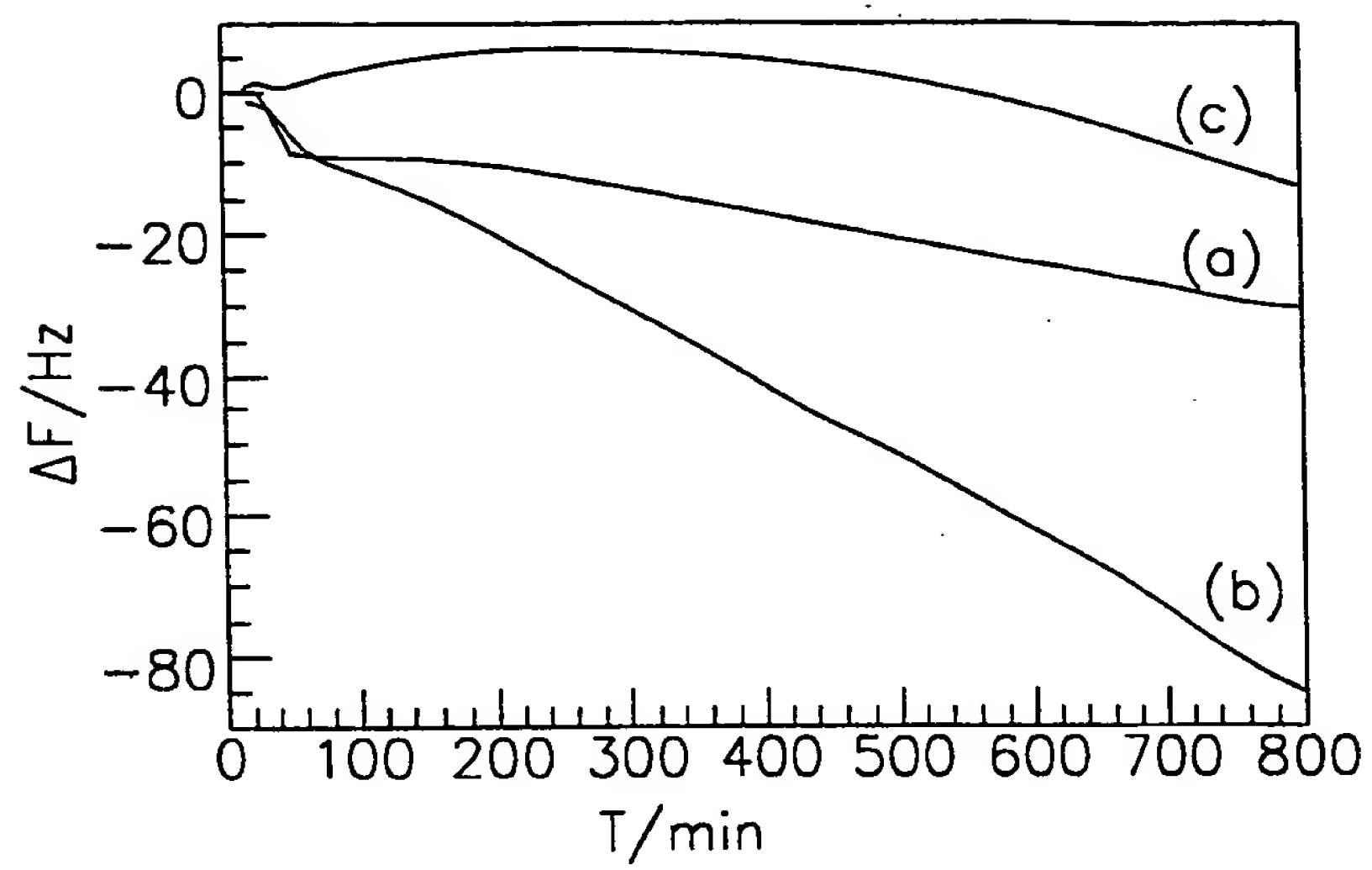


FIG.25

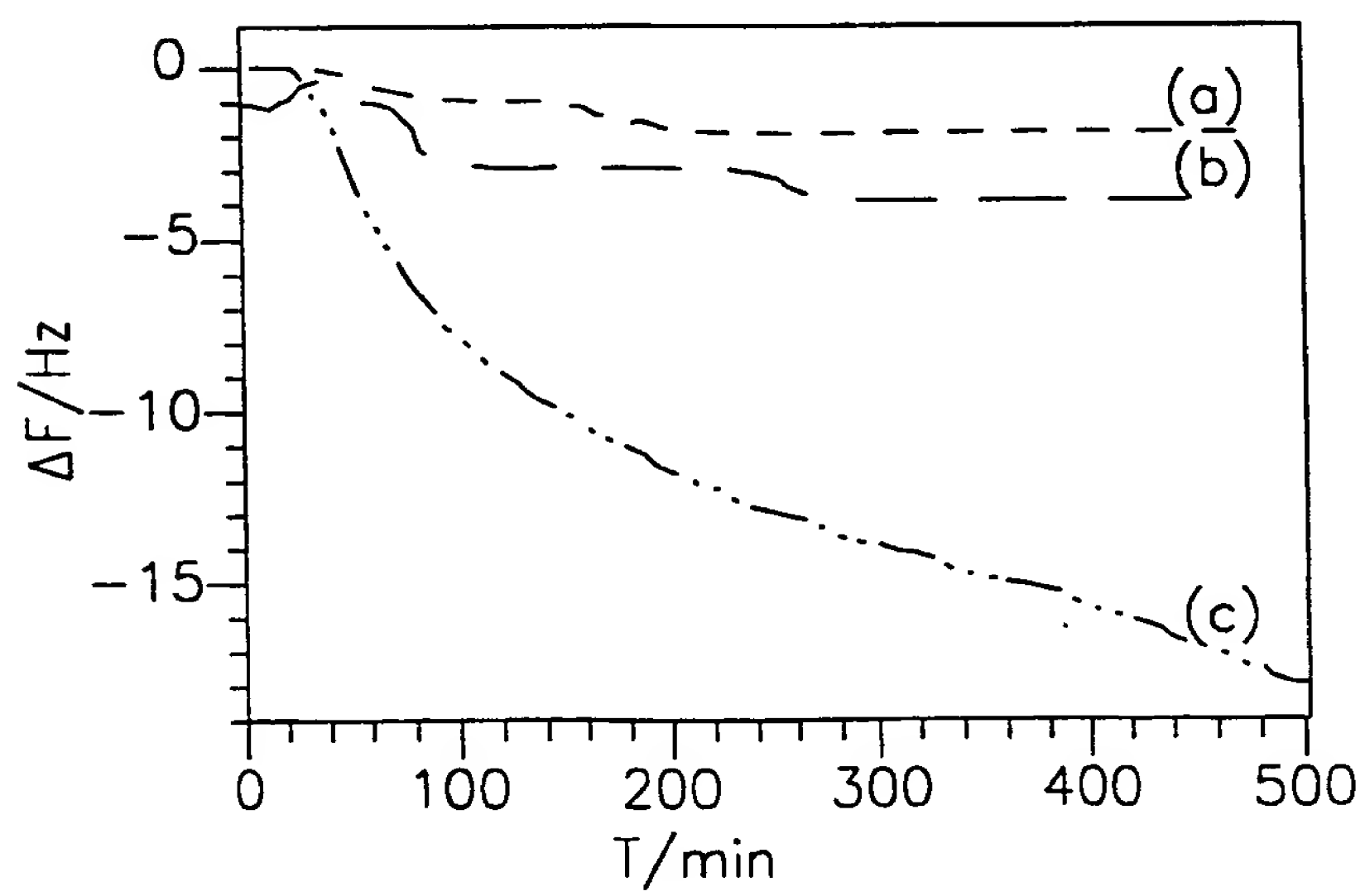


FIG.26

22/29

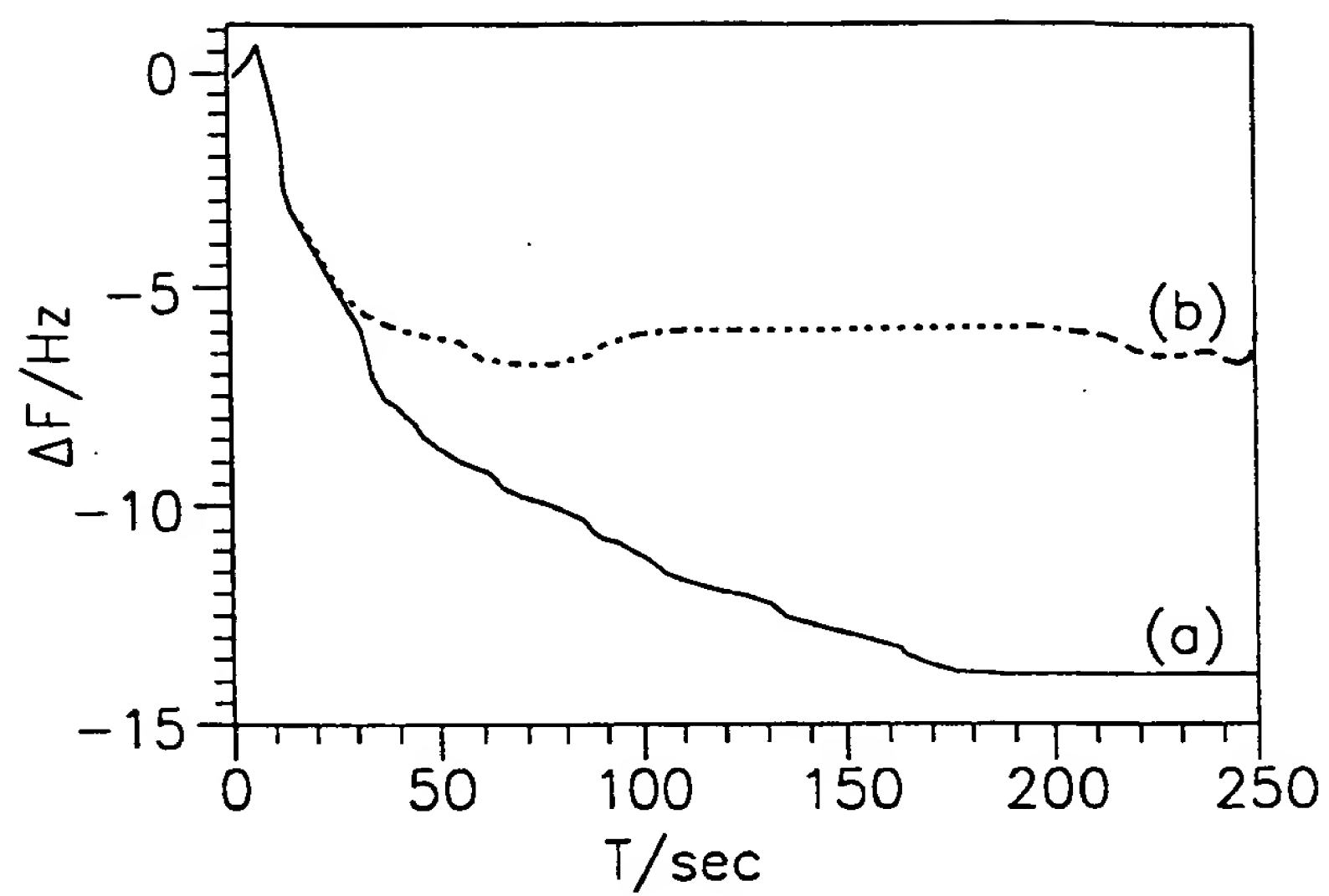


FIG.27A

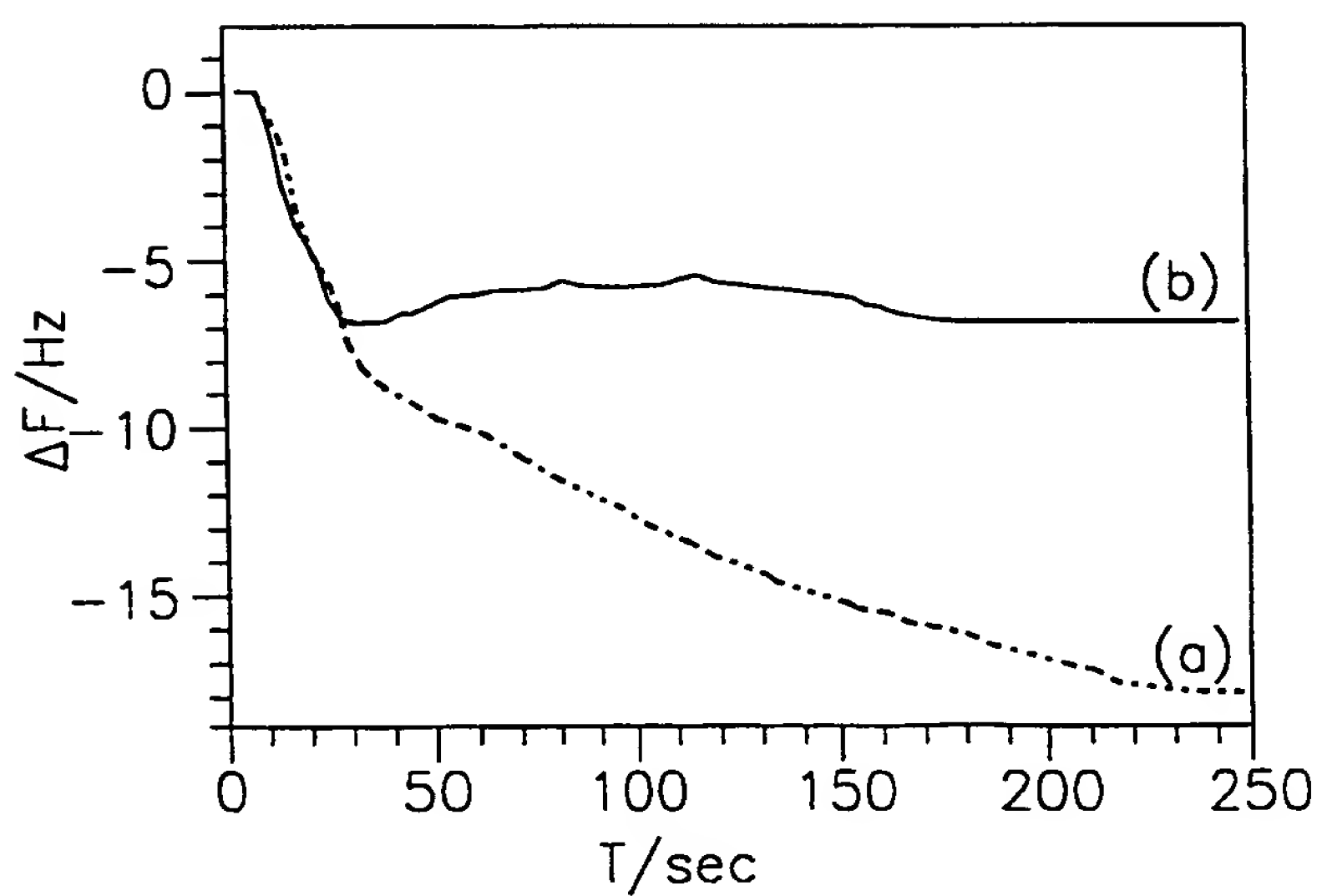


FIG.27B

23/29

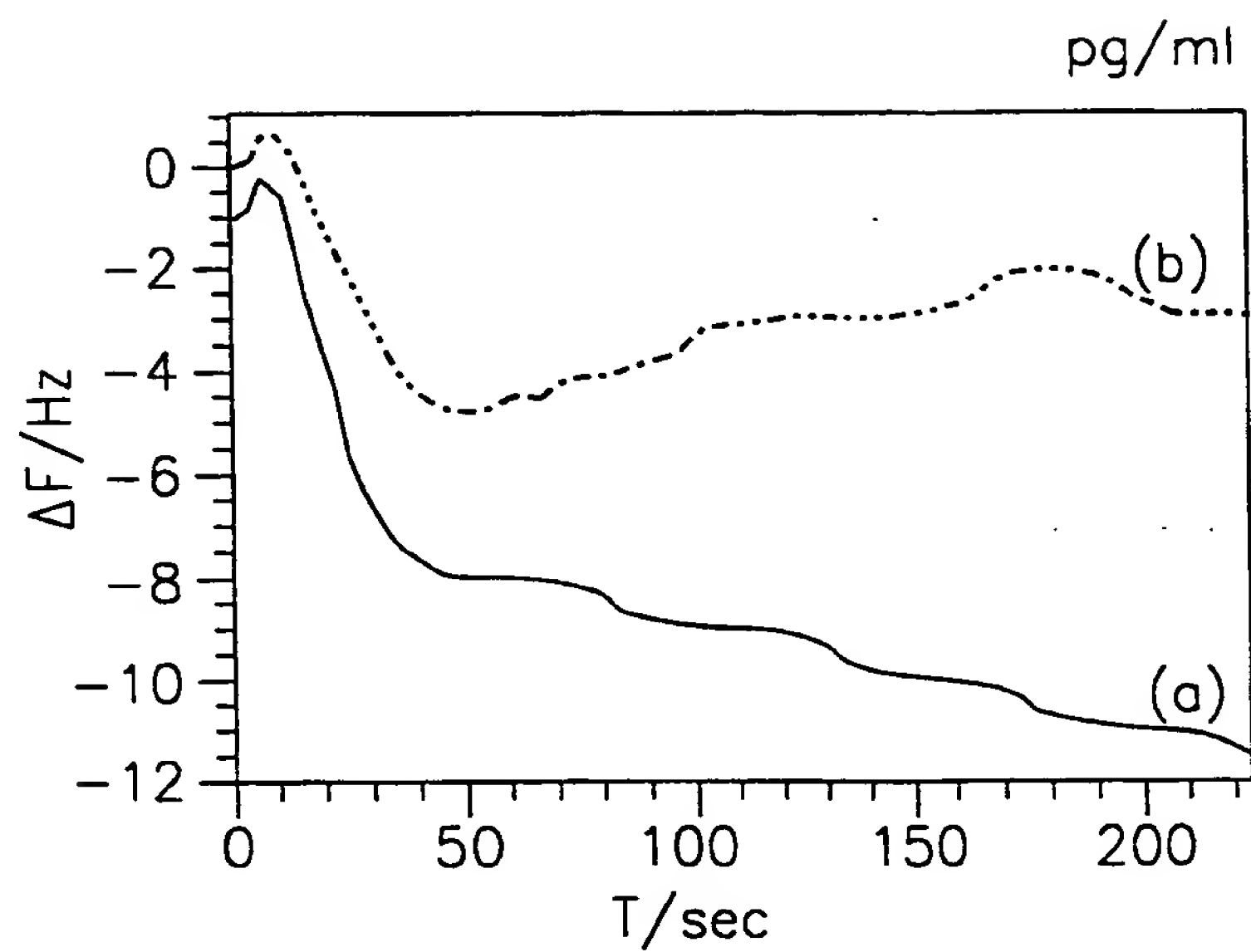


FIG.27C

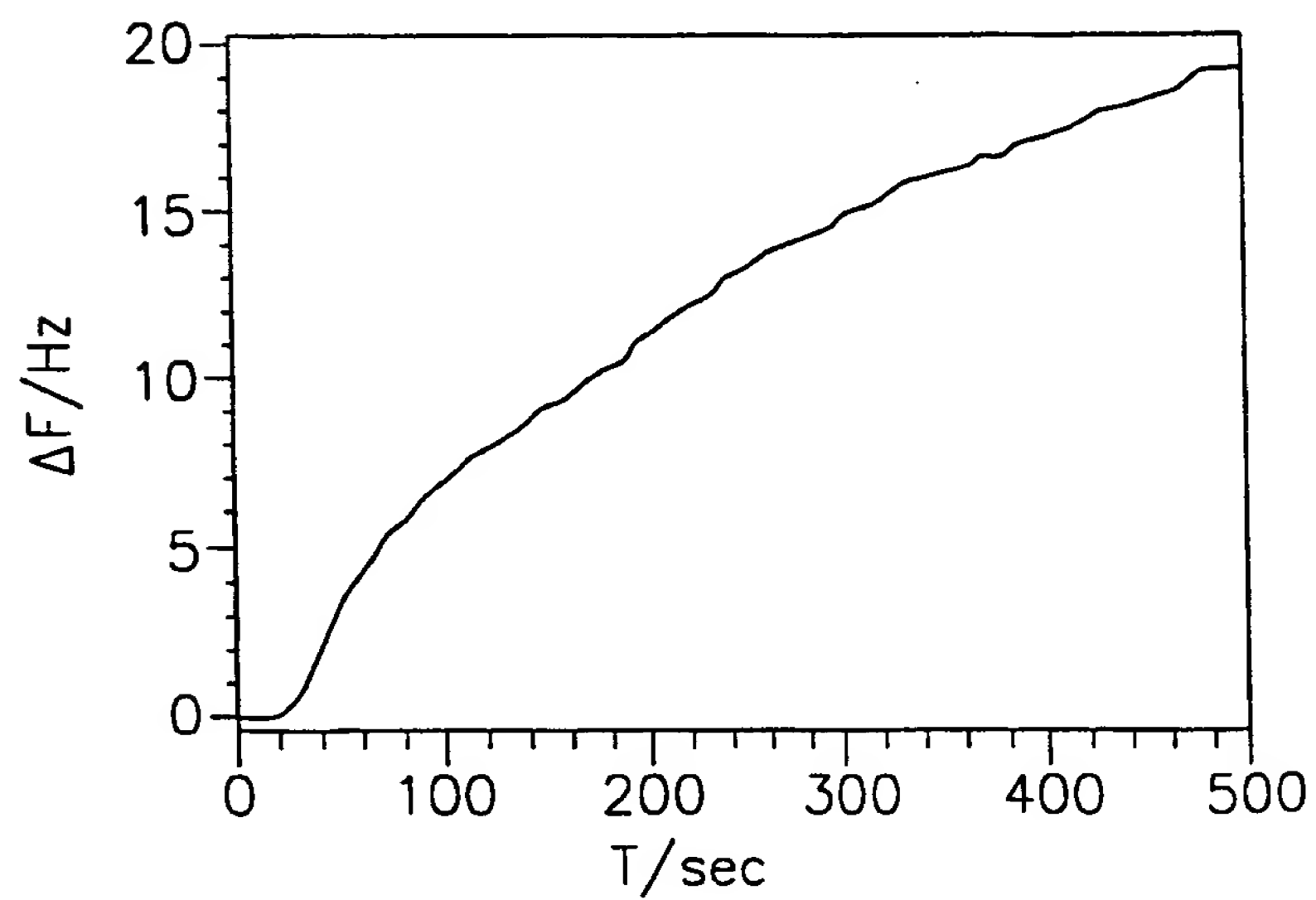


FIG.28A

24/29

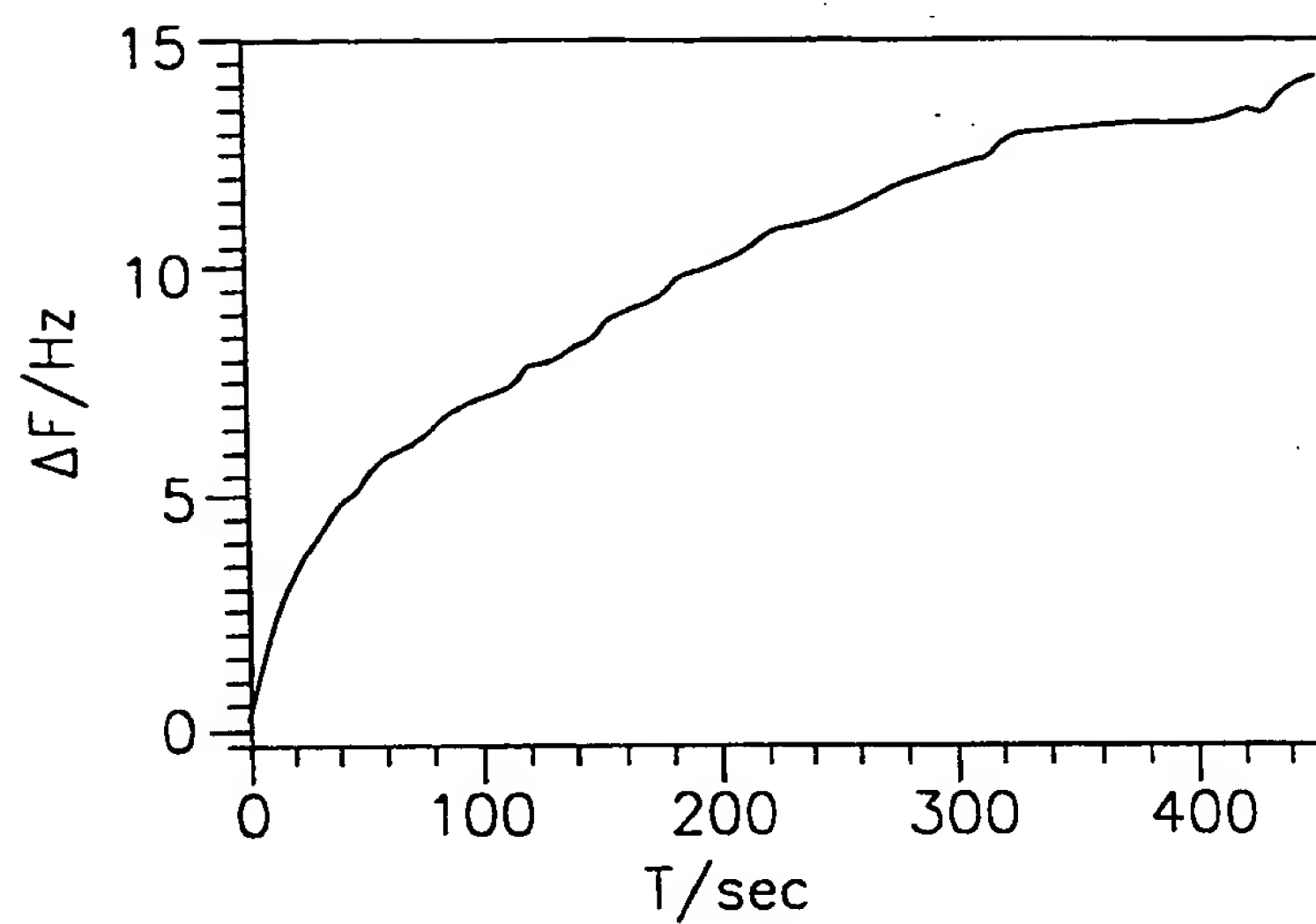


FIG.28B

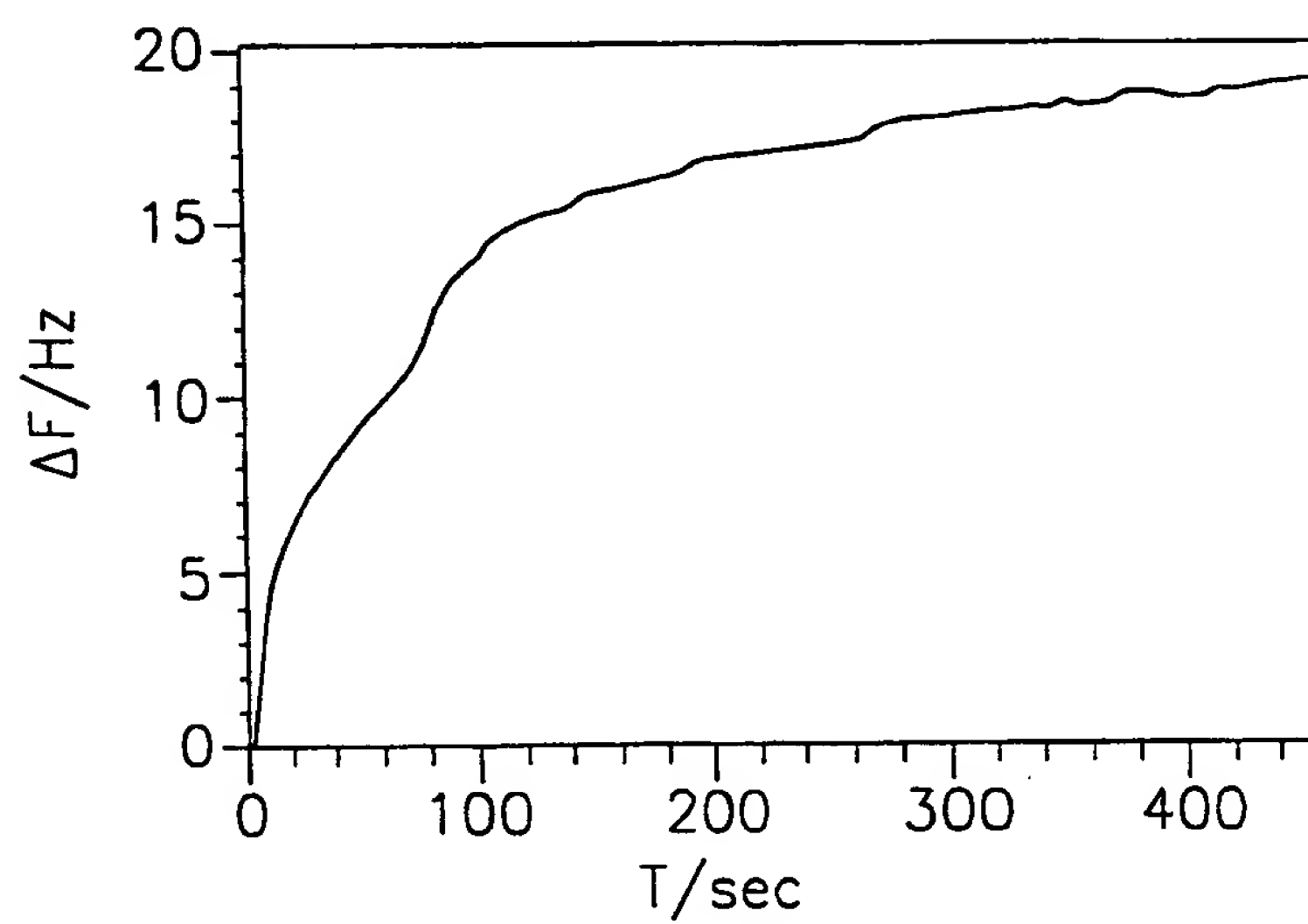


FIG.28C

25/29

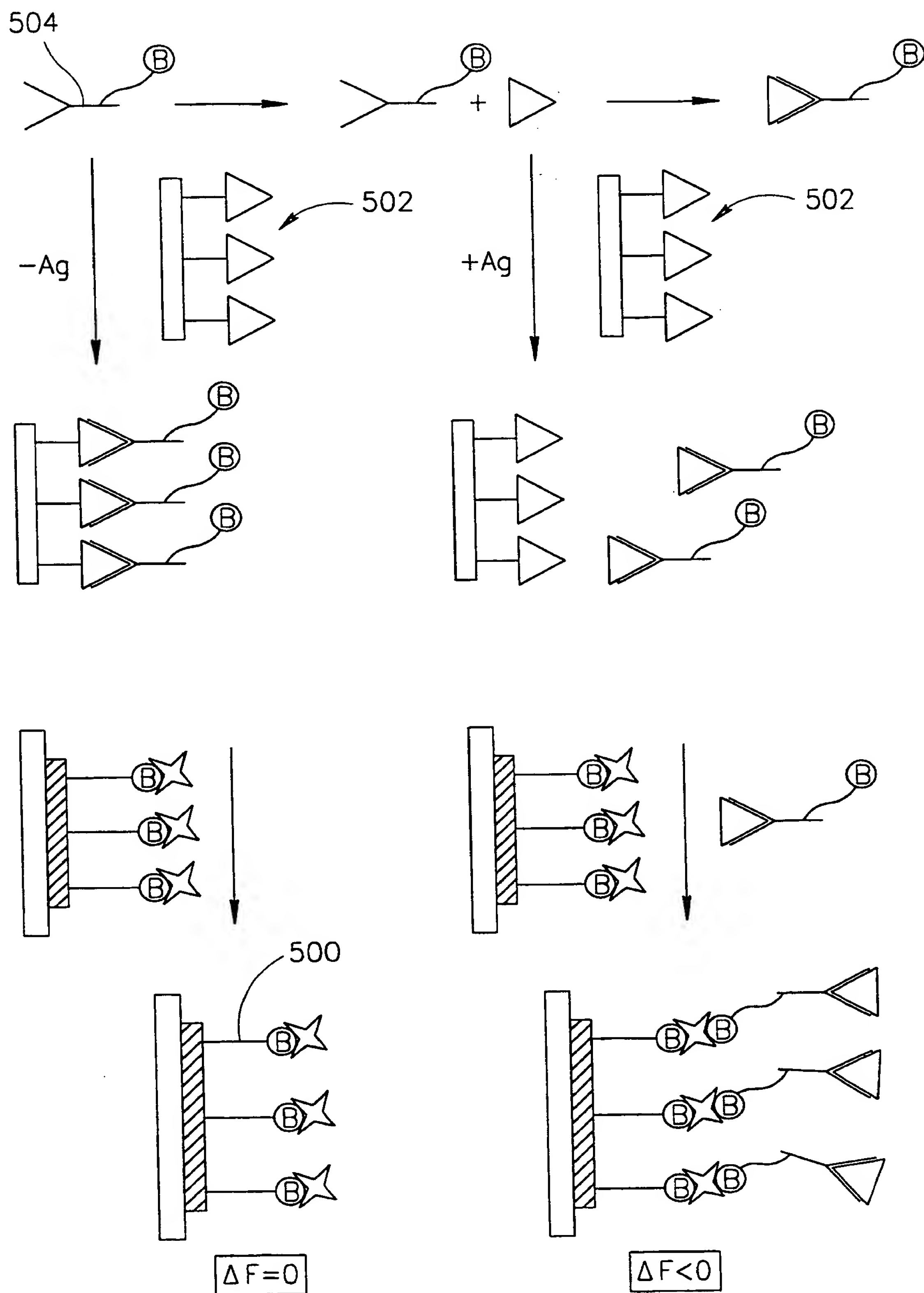


FIG.29

26/29

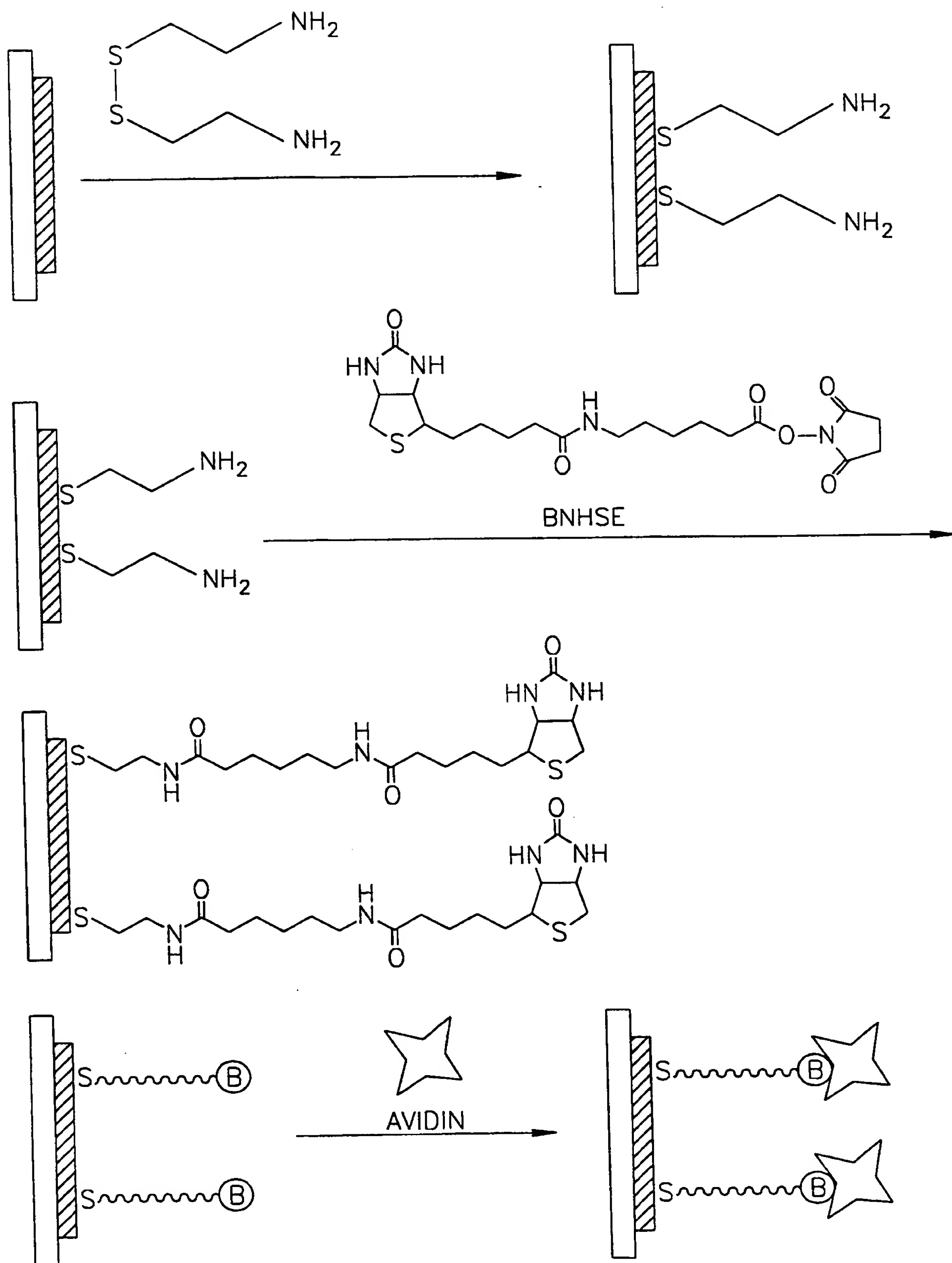


FIG.30

27/29

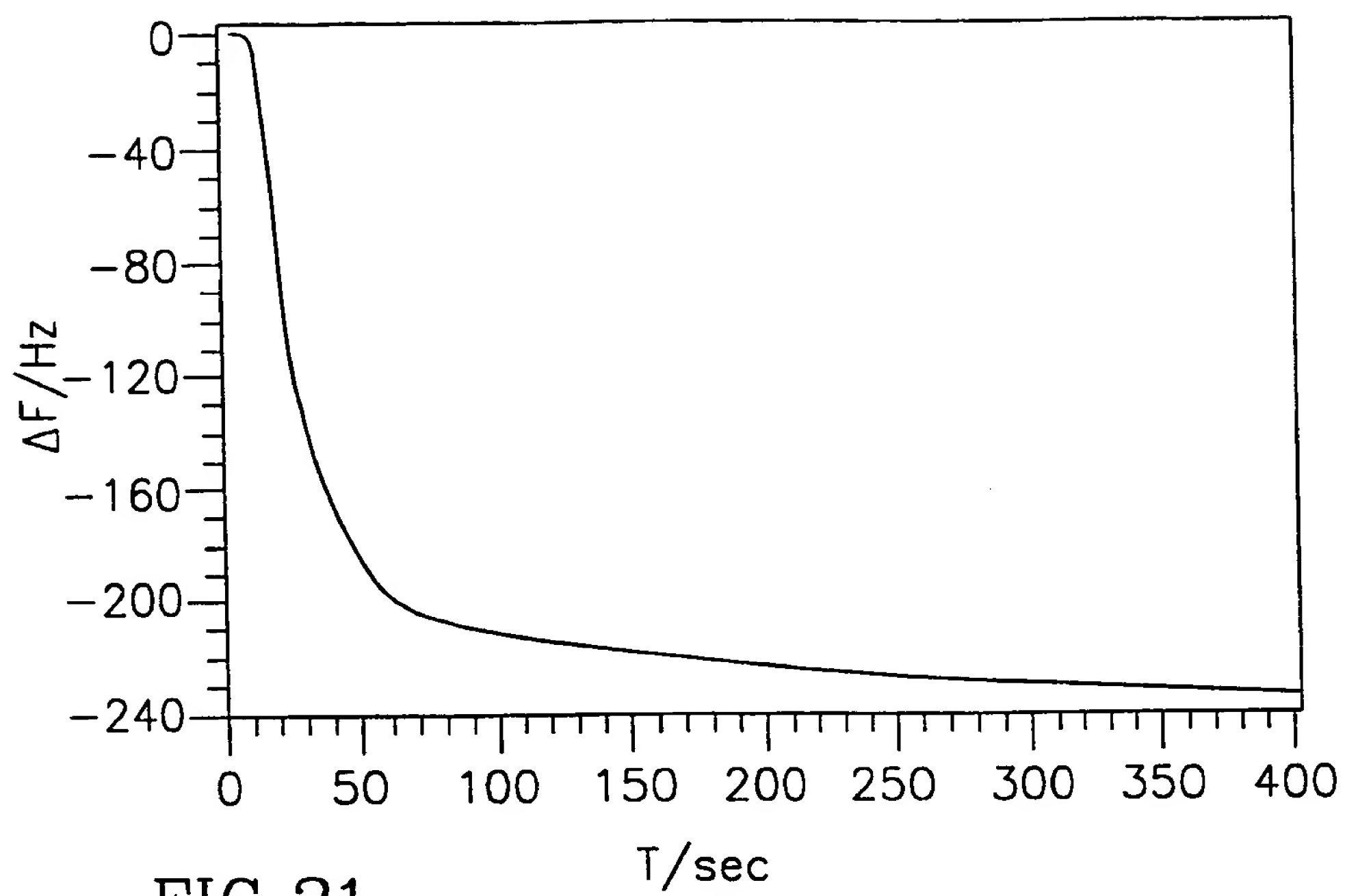


FIG.31

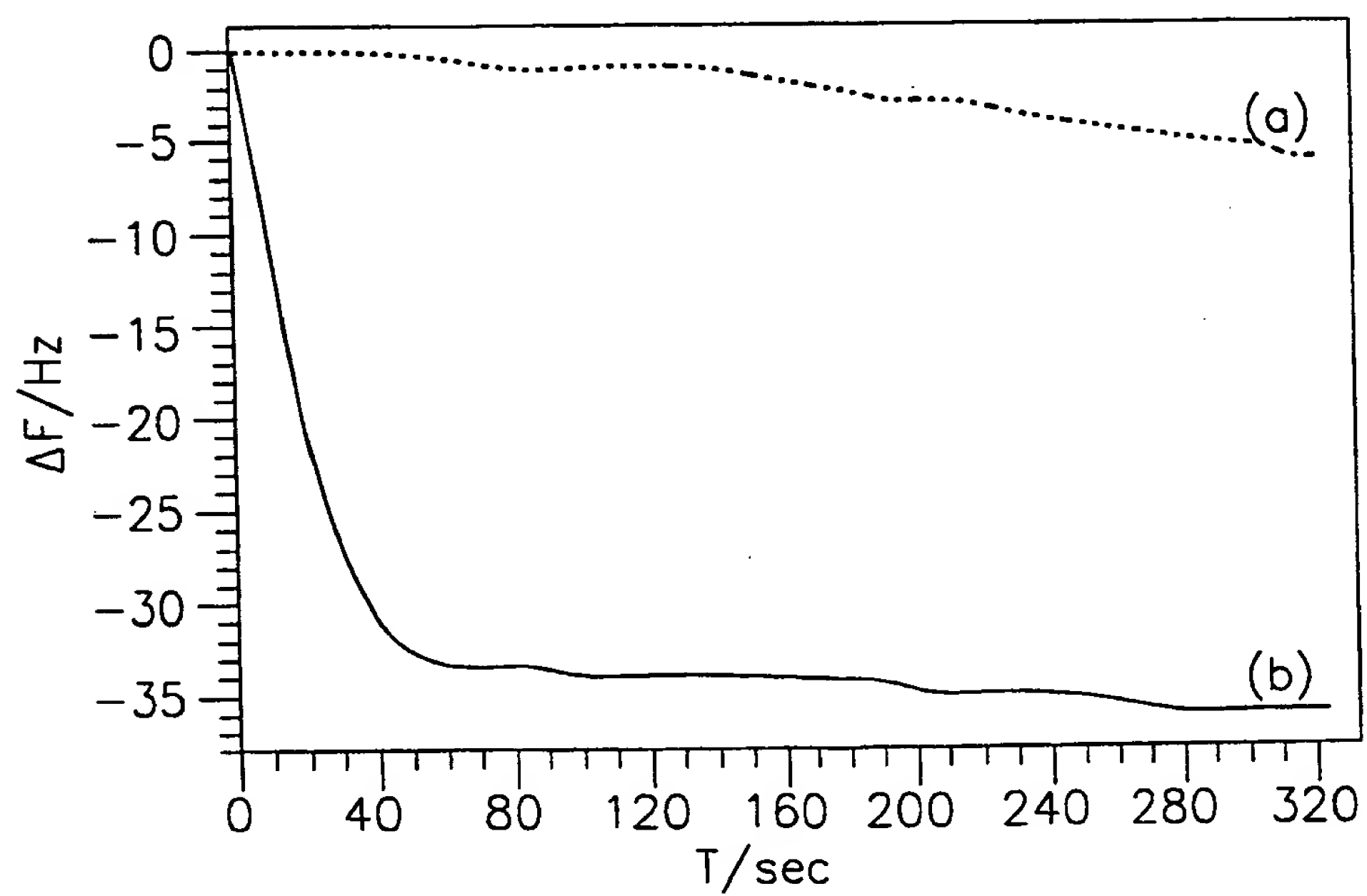


FIG.32

28/29

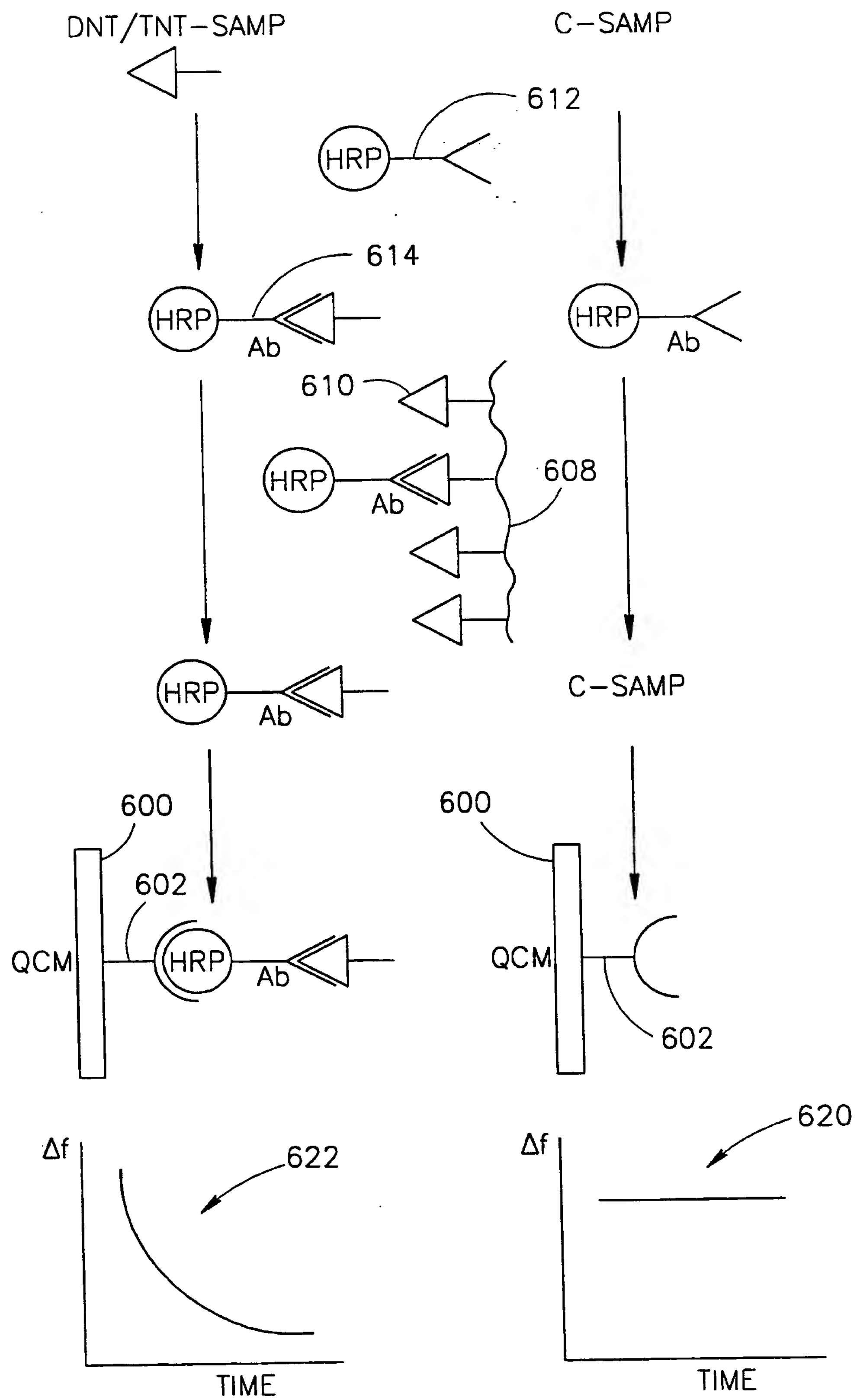


FIG.33

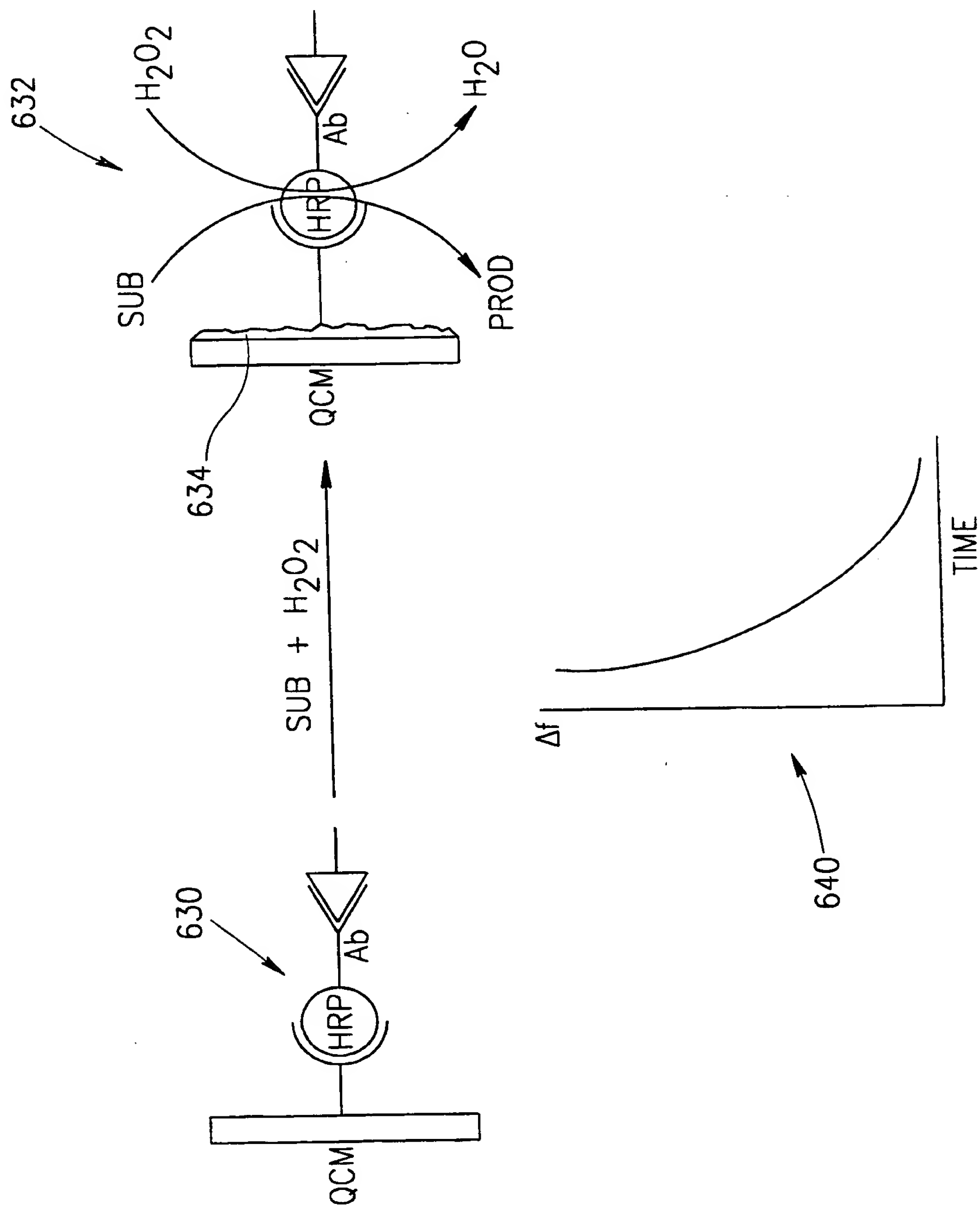


FIG.34

SEQUENCE LISTING

<110> YEDA RESEARCH AND DEVELOPMENT COMPANY LTD.

YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW

<120> DETECTION OF SMALL MOLECULES BY USE OF A PIEZOELECTRIC
SENSOR

<130> 122177 YISSUM + YEDA

<140>

<141>

<150> IL128212

<151> 1999-01-25

<160> 8

<170> PatentIn Ver. 2.1

<210> 1

<211> 37

<212> DNA

<213> Humanus

<400> 1

cccgtctaga ggagayatyg twatgaccca gtctcca

37

<210> 2

<211> 22

<212> DNA

<213> Humanus

<400> 2

gtttkatctc gagcttkgts cc

22

<210> 3

<211> 17

<212> DNA

<213> Humanus

<400> 3

aggtsmarct kctcgag

17

<210> 4

<211> 34
 <212> DNA
 <213> Humanus

<400> 4
 tgmrgagacg gtgaccgtrg tyccttggcc ccag

34

<210> 5
 <211> 408
 <212> DNA
 <213> Humanus

<400> 5
 atgagagtgc tgattctttt gtggctgttc acagcctttc ctggtatcct gtctgatgtg 60
 cagcttcagg agtcgggacc tggcctgggt aagccttccc agtctctgtc cctcacctgc 120
 tctgtcactg gttactcaat caccagtggg tatgcctgga actggatccg gcagtttcca 180
 ggaaacaaac tggagtggat gggctacata agctacagtg gtttcactag ctacaaccca 240
 tctctcagaa gtcgaatctc tttcactcga gacacatcca agaaccagtt cttcctgcag 300
 ttgaattctg tgacttctga ggacacagcc acatattact gtgcaagatg ggactacggg 360
 actacctacg ggtacttcga tgtctggggc caagggacta cggtcacc 408

<210> 6
 <211> 136
 <212> PRT
 <213> Humanus

<400> 6
 Met Arg Val Leu Ile Leu Leu Trp Leu Phe Thr Ala Phe Pro Gly Ile
 1 5 10 15
 Leu Ser Asp Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro
 20 25 30
 Ser Gln Ser Leu Ser Leu Thr Cys Ser Val Thr Gly Tyr Ser Ile Thr
 35 40 45
 Ser Gly Tyr Ala Trp Asn Trp Ile Arg Gln Phe Pro Gly Asn Lys Leu
 50 55 60
 Glu Trp Met Gly Tyr Ile Ser Tyr Ser Gly Phe Thr Ser Tyr Asn Pro
 65 70 75 80
 Ser Leu Arg Ser Arg Ile Ser Phe Thr Arg Asp Thr Ser Lys Asn Gln
 85 90 95
 Phe Phe Leu Gln Leu Asn Ser Val Thr Ser Glu Asp Thr Ala Thr Tyr

100 105 110
 Tyr Cys Ala Arg Trp Asp Tyr Gly Thr Thr Tyr Gly Tyr Phe Asp Val
 115 120 125

Trp Gly Gln Gly Thr Thr Val Thr
 130 135

<210> 7
 <211> 348
 <212> DNA
 <213> Humanus

<400> 7
 tctagaggag atatcggttat gaccagctct ccatacctccc tgagtgtgtc agcaggagag 60
 aaggctacta tgagctgcaa gtccagtcag agtctgttaa acagtagaaa tcaaaagaac 120
 tacttggcct ggtaccagca gaaaccagga cagcctccta aacttttgat ctacggggta 180
 tttattagggt attctggggt ccctgatcgc ttcacaggca gtggatctgg aaccgatttc 240
 actcttacca tcagcagtgt gcaggctgaa gacctggcag tttattactg tcagaatgat 300
 catatttatc cgtacacgtt cggagggggg accaagctgg aaataaaa 348

<210> 8
 <211> 116
 <212> PRT
 <213> Humanus

<400> 8
 Ser Arg Gly Asp Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ser Val
 1 5 10 15
 Ser Ala Gly Glu Lys Val Thr Met Ser Cys Lys Ser Ser Gln Ser Leu
 20 25 30
 Leu Asn Ser Arg Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys
 35 40 45
 Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Gly Val Phe Ile Arg Asp
 50 55 60
 Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe
 65 70 75 80
 Thr Leu Thr Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr
 85 90 95

Cys Gln Asn Asp His Ile Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys
 100 105 110

Leu Glu Ile Lys
 115

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
27 July 2000 (27.07.2000)

PCT

(10) International Publication Number
WO 00/43774 A3

(51) International Patent Classification⁷: G01N 33/53,
33/543, C07K 16/44

(74) Agent: REINHOLD COHN AND PARTNERS; P.O.
Box 4060, 61040 Tel Aviv (IL).

(21) International Application Number: PCT/IL00/00048

(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(22) International Filing Date: 25 January 2000 (25.01.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
128212 25 January 1999 (25.01.1999) IL

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(71) Applicants (*for all designated States except US*): YIS-
SUM RESEARCH DEVELOPMENT COMPANY
OF THE HEBREW UNIVERSITY OF JERUSALEM
[IL/IL]; Jabotinsky Street 46, 92182 Jerusalem (IL).
YEDA RESEARCH AND DEVELOPMENT COM-
PANY LTD. [IL/IL]; P.O. Box 95, 76100 Rehovot (IL).

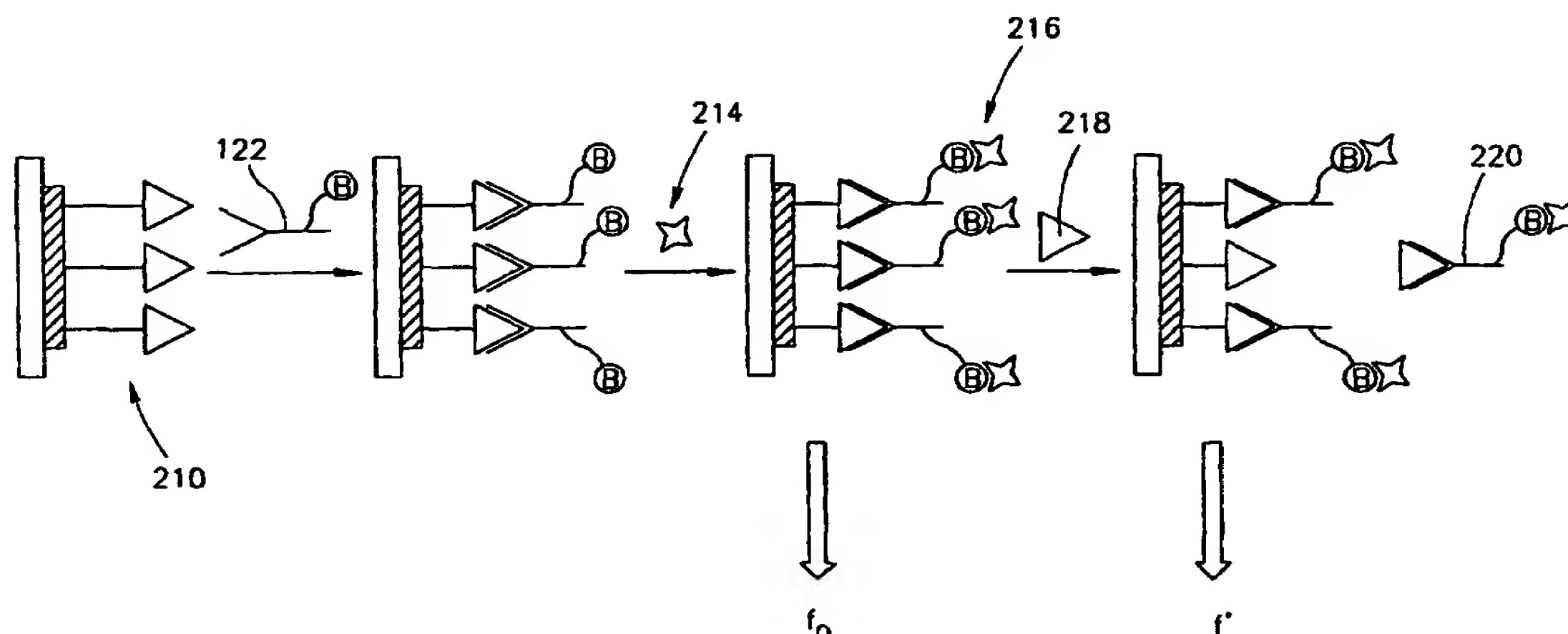
Published:
— With international search report.

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): WILLNER, Itamar
[IL/IL]; Hashalom Street 12, 90805 Mevaseret Zion (IL).
ESHAR, Zelig [IL/IL]; Hess Street 18, 76346 Rehovot
(IL).

(88) Date of publication of the international search report:
1 March 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: DETECTION OF SMALL MOLECULES BY USE OF A PIEZOELECTRIC SENSOR



(57) Abstract: Apparatus and method of detecting assayed molecules in a sample make use of a sensing member comprising a piezoelectric crystal with a sensing surface that can interact with a medium in contact therewith by either binding a first indicator agent from the medium, or by releasing a second indicator agent originally immobilized on the sensing surface into the medium. The binding or release causes a change in mass detectable by the use of an electric or electronic utility, through a change in resonance frequency. The medium interacting with the sensing member is a treated sample preparation obtained by reacting the sample with one or both of a reagent solution or sample-processing hardware, such that said medium comprises a first indicator agent or a second indicator agent-releasing species at a concentration of said agent or species which is in correlation to the concentration of the assayed molecules in the sample.

WO 00/43774 A3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 00/00048

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/53 G01N33/543 C07K16/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

PAJ, EPO-Internal, BIOSIS, WPI Data, CHEM ABS Data, MEDLINE, EMBASE, SCISEARCH, COMPENDEX, STRAND, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 04314 A (YISSUM RES DEV CO ; LEVI SHLOMO (IL); COHEN Yael (IL); DAGAN ARIE () 6 February 1997 (1997-02-06)	1,3-9, 12-15, 17,19, 20,43, 46,47, 49,51,52
Y	page 24, line 3 -page 26, line 26 page 33, line 4 -page 34, line 21 examples 2,6	1,13,14, 35-38, 41-43, 67-70, 72,73

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

18 October 2000

Date of mailing of the international search report

31.10.2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Nooij, F

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 00/00048

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 658 732 A (EBERSOLE RICHARD CALVIN ET AL) 19 August 1997 (1997-08-19) column 5, line 1 - line 35 claims	1,13,14, 35-38, 41-43, 67-70, 72,73
X	BRINK S -A: "Bofors Schnauzer-a biosensor for detection of explosives" EUREL INTERNATIONAL CONFERENCE. THE DETECTION OF ABANDONED LAND MINES: A HUMANITARIAN IMPERATIVE SEEKING A TECHNICAL SOLUTION,XX,XX, 7 October 1996 (1996-10-07), pages 33-36, XP002136334 page 34, column 1, paragraph 5 -page 34, column 2, paragraph 2	1-5,9, 12-18, 43-46
X	WO 98 57141 A (SAMUELSSON MAGNUS ;ALM ARNE (SE); ANDERSSON CLAES (SE); ARNELL JOH) 17 December 1998 (1998-12-17) page 4, line 24 -page 6, line 21	1-5,9, 12-18, 43-46
X	EP 0 453 224 A (HUGHES AIRCRAFT CO) 23 October 1991 (1991-10-23) abstract column 2, line 20 - line 31	1-5,9, 12-18, 43-46
A		74-78
A	US 4 999 284 A (WARD MICHAEL D ET AL) 12 March 1991 (1991-03-12) column 2, line 47 -column 3, line 39	1,13,14, 35-43, 67-73
A	PATENT ABSTRACTS OF JAPAN vol. 014, no. 034 (C-679), 23 January 1990 (1990-01-23) & JP 01 269485 A (MATSUSHITA ELECTRIC IND CO LTD), 26 October 1989 (1989-10-26) abstract	74-78
A	PATENT ABSTRACTS OF JAPAN vol. 014, no. 034 (C-679), 23 January 1990 (1990-01-23) & JP 01 269488 A (MATSUSHITA ELECTRIC IND CO LTD), 26 October 1989 (1989-10-26) abstract	74-78

-/-

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 00/00048

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	D. FETTEROLF ET AL.: "An enzyme-linked immunosorbent assay (ELISA) for trinitrotoluene (TNT) residue on hands." JOURNAL OF FORENSIC SCIENCES, vol. 36, no. 2, March 1991 (1991-03), pages 343-349, XP000946955 Philadelphia, PA, USA abstract	74-78
A	U. NARANG ET AL.: "Multianalyte detection using a capillary-based flow immunosensor." ANALYTICAL BIOCHEMISTRY, vol. 255, no. 1, 1 January 1998 (1998-01-01), pages 13-19, XP002150368 abstract figure 1 page 13, right-hand column, line 4 - line 19	74-78

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL 00/00048

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

1-20, 35-52, 67-78
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1,13,14,43 (all in part) and 2-12,15-20,
44-52 (all entirely)

Apparatus and methods for detecting explosives providing:
- a sensing surface carrying moieties or residues of TNT or DNT bound to an antibody.

2. Claims: 1,13,14,43 (all in part) and 21-26,
53-58 (all entirely)

Apparatus and methods for detecting explosives providing:
- a sensing surface carrying moieties or residues of TNT or DNT,
- a reagent for mixing with the sample prior to introduction in the cell comprising an antibody.

3. Claims: 1,13,14,43 (all in part) and 27-34,
59-66 (all entirely)

Apparatus and methods for detecting explosives providing:
- a sensing surface carrying moieties or residues of TNT or DNT,
- a reagent for mixing with the sample prior to introduction in the cell comprising an antibody,
- a filtration system to remove unbound antibodies prior to introduction in the cell.

4. Claims: 1,13,14,43 (all in part) and 35-42,
67-73 (all entirely)

Apparatus and methods for detecting explosives providing:
- a reagent for mixing with the sample prior to introduction in the cell comprising an antibody conjugated to an enzyme,
- a sensing surface carrying an antibody capable of binding the enzyme,
- a filtration system to remove unbound Antibody-Enzyme complexes,
- Further reagents reacting with the enzyme to form an insoluble product.

5. Claims: 74-78

A protein (antibody) able to bind TNT or DNT described as for use in a piezo electric micro-gravimetric assay.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IL 00/00048

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9704314 A	06-02-1997	AU 6317896 A EP 0843816 A JP 11510247 T	18-02-1997 27-05-1998 07-09-1999
US 5658732 A	19-08-1997	CA 2066643 A DE 69010506 D DE 69010506 T EP 0494896 A JP 5500715 T WO 9105261 A	05-04-1991 11-08-1994 22-12-1994 22-07-1992 12-02-1993 18-04-1991
WO 9857141 A	17-12-1998	AU 8626198 A EP 0988519 A NO 996160 A SE 9702407 A	30-12-1998 29-03-2000 19-01-2000 25-12-1998
EP 0453224 A	23-10-1991	US 5179028 A CA 2039081 A FI 911926 A JP 4230860 A KR 9402465 B NO 911531 A	12-01-1993 21-10-1991 21-10-1991 19-08-1992 24-03-1994 21-10-1991
US 4999284 A	12-03-1991	CA 1332221 A DE 68916423 D DE 68916423 T EP 0408578 A JP 3503567 T WO 8909937 A	04-10-1994 28-07-1994 24-11-1994 23-01-1991 08-08-1991 19-10-1989
JP 01269485 A	26-10-1989	NONE	
JP 01269488 A	26-10-1989	NONE	

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 122177.9 LK	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/IL 00/ 00048	International filing date (day/month/year) 25/01/2000	(Earliest) Priority Date (day/month/year) 25/01/1999
Applicant YISSUM RESEARCH DEVELOPMENT COMPANY OF THE...et al		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 7 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☒ contained in the international application in written form.

☒ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☒ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 16

☐ as suggested by the applicant.

☒ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☐ None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL 00/00048

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

1-20, 35-52, 67-78
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1,13,14,43 (all in part) and 2-12,15-20,
44-52 (all entirely)

Apparatus and methods for detecting explosives providing:
- a sensing surface carrying moieties or residues of TNT or DNT bound to an antibody.

2. Claims: 1,13,14,43 (all in part) and 21-26,
53-58 (all entirely)

Apparatus and methods for detecting explosives providing:
- a sensing surface carrying moieties or residues of TNT or DNT,
- a reagent for mixing with the sample prior to introduction in the cell comprising an antibody.

3. Claims: 1,13,14,43 (all in part) and 27-34,
59-66 (all entirely)

Apparatus and methods for detecting explosives providing:
- a sensing surface carrying moieties or residues of TNT or DNT,
- a reagent for mixing with the sample prior to introduction in the cell comprising an antibody,
- a filtration system to remove unbound antibodies prior to introduction in the cell.

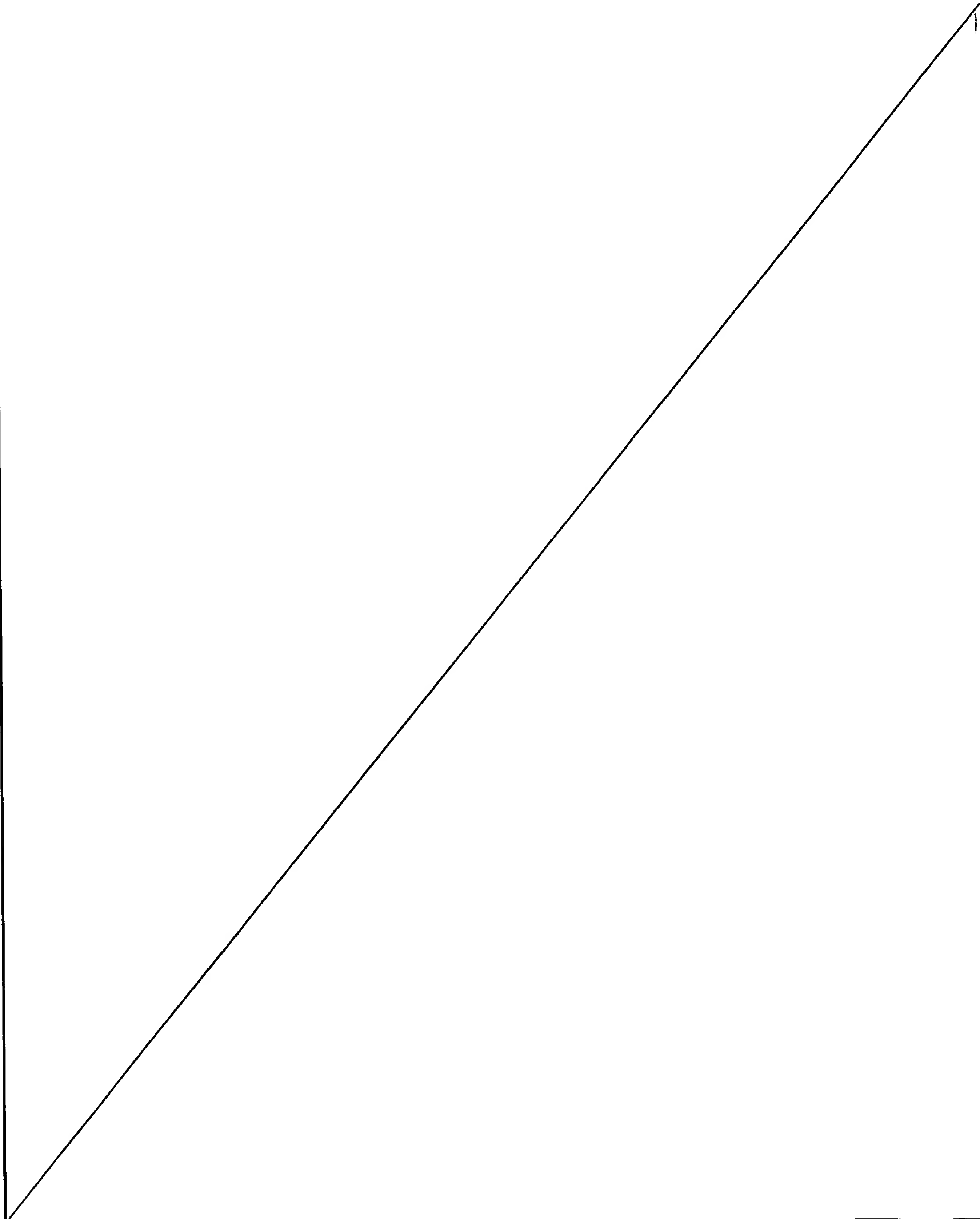
4. Claims: 1,13,14,43 (all in part) and 35-42,
67-73 (all entirely)

Apparatus and methods for detecting explosives providing:
- a reagent for mixing with the sample prior to introduction in the cell comprising an antibody conjugated to an enzyme,
- a sensing surface carrying an antibody capable of binding the enzyme,
- a filtration system to remove unbound Antibody-Enzyme complexes,
- Further reagents reacting with the enzyme to form an insoluble product.

5. Claims: 74-78

A protein (antibody) able to bind TNT or DNT described as for use in a piezo electric micro-gravimetric assay.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210



INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 00/00048

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/53 G01N33/543 C07K16/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

PAJ, EPO-Internal, BIOSIS, WPI Data, CHEM ABS Data, MEDLINE, EMBASE, SCISEARCH, COMPENDEX, STRAND, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 04314 A (YISSUM RES DEV CO ;LEVI SHLOMO (IL); COHEN Yael (IL); DAGAN ARIE () 6 February 1997 (1997-02-06)	1,3-9, 12-15, 17,19, 20,43, 46,47, 49,51,52
Y	page 24, line 3 -page 26, line 26 page 33, line 4 -page 34, line 21 examples 2,6 --- -/--	1,13,14, 35-38, 41-43, 67-70, 72,73

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

18 October 2000

Date of mailing of the international search report

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Nooij, F

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 00/00048

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 658 732 A (EBERSOLE RICHARD CALVIN ET AL) 19 August 1997 (1997-08-19) column 5, line 1 - line 35 claims ---	1,13,14, 35-38, 41-43, 67-70, 72,73
X	BRINK S -A: "Bofors Schnauzer-a biosensor for detection of explosives" EUREL INTERNATIONAL CONFERENCE. THE DETECTION OF ABANDONED LAND MINES: A HUMANITARIAN IMPERATIVE SEEKING A TECHNICAL SOLUTION,XX,XX, 7 October 1996 (1996-10-07), pages 33-36, XP002136334 page 34, column 1, paragraph 5 -page 34, column 2, paragraph 2 ---	1-5,9, 12-18, 43-46
X	WO 98 57141 A (SAMUELSSON MAGNUS ;ALM ARNE (SE); ANDERSSON CLAES (SE); ARNELL JOH) 17 December 1998 (1998-12-17) page 4, line 24 -page 6, line 21 ---	1-5,9, 12-18, 43-46
X	EP 0 453 224 A (HUGHES AIRCRAFT CO) 23 October 1991 (1991-10-23) abstract column 2, line 20 - line 31 ---	1-5,9, 12-18, 43-46
A		74-78
A	US 4 999 284 A (WARD MICHAEL D ET AL) 12 March 1991 (1991-03-12) column 2, line 47 -column 3, line 39 ---	1,13,14, 35-43, 67-73
A	PATENT ABSTRACTS OF JAPAN vol. 014, no. 034 (C-679), 23 January 1990 (1990-01-23) & JP 01 269485 A (MATSUSHITA ELECTRIC IND CO LTD), 26 October 1989 (1989-10-26) abstract ---	74-78
A	PATENT ABSTRACTS OF JAPAN vol. 014, no. 034 (C-679), 23 January 1990 (1990-01-23) & JP 01 269488 A (MATSUSHITA ELECTRIC IND CO LTD), 26 October 1989 (1989-10-26) abstract ---	74-78

	-/--	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 00/00048

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	D. FETTEROLF ET AL.: "An enzyme-linked immunosorbent assay (ELISA) for trinitrotoluene (TNT) residue on hands." JOURNAL OF FORENSIC SCIENCES, vol. 36, no. 2, March 1991 (1991-03), pages 343-349, XP000946955 Philadelphia, PA, USA abstract	74-78
A	U. NARANG ET AL.: "Multianalyte detection using a capillary-based flow immunosensor." ANALYTICAL BIOCHEMISTRY, vol. 255, no. 1, 1 January 1998 (1998-01-01), pages 13-19, XP002150368 abstract figure 1 page 13, right-hand column, line 4 - line 19	74-78

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IL 00/00048

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9704314 A	06-02-1997	AU 6317896 A EP 0843816 A JP 11510247 T	18-02-1997 27-05-1998 07-09-1999
US 5658732 A	19-08-1997	CA 2066643 A DE 69010506 D DE 69010506 T EP 0494896 A JP 5500715 T WO 9105261 A	05-04-1991 11-08-1994 22-12-1994 22-07-1992 12-02-1993 18-04-1991
WO 9857141 A	17-12-1998	AU 8626198 A EP 0988519 A NO 996160 A SE 9702407 A	30-12-1998 29-03-2000 19-01-2000 25-12-1998
EP 0453224 A	23-10-1991	US 5179028 A CA 2039081 A FI 911926 A JP 4230860 A KR 9402465 B NO 911531 A	12-01-1993 21-10-1991 21-10-1991 19-08-1992 24-03-1994 21-10-1991
US 4999284 A	12-03-1991	CA 1332221 A DE 68916423 D DE 68916423 T EP 0408578 A JP 3503567 T WO 8909937 A	04-10-1994 28-07-1994 24-11-1994 23-01-1991 08-08-1991 19-10-1989
JP 01269485 A	26-10-1989	NONE	
JP 01269488 A	26-10-1989	NONE	